THE ROLE OF THE AUTOPHAGY-LYSOSOME PATHWAY IN IN VITRO MODELS OF NEURODEGENERATION

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MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Recent studies have provided strong evidence that alterations in protein degradation pathways, such as the autophagy-lysosome pathway (ALP), may contribute to neuronal dysfunction and death which lead to clinical symptoms diagnosed with various neurodegenerative diseases. Parkinson’s disease (PD) is characterized by neuromuscular abnormalities resulting from the pathological loss of substantia nigra dopaminergic neurons and widespread detection of Lewy bodies, intracellular protein inclusions composed primarily of \(\alpha\)-synuclein. Additionally, altered gene function regulating the expression of \(\alpha\)-synuclein has been directly linked to the PD pathogenesis.

Autophagy is an intracellular degradation process that when altered can lead to the accumulation neurotoxic proteins such as \(\alpha\)-synuclein. Experimental inhibition of the ALP in models of PD is effective at reproducing \(\alpha\)-synuclein accumulation and cell death. Conversely, stimuli that enhance ALP function are protective in PD and other models of neurodegenerative disease. Macroautophagy is a subset of autophagy that regulates degradation by sequestering cytosolic material within double-membraned vesicles, or autophagosomes, that are ultimately degraded by the lysosome. The vacuolar type-ATPase (V-ATPase) regulates acidic vesicle pH, and its inhibition by bafilomycin A1 (BafA1) is mediated through its interaction the V-ATPase subunit ATP6V0C. BafA1-mediated inhibition of V-ATPase leads to elevated lysosome pH, altered autophagy, accumulation of \(\alpha\)-synuclein, and cell death.
While autophagy is known to be important in maintenance of neuronal cell function, its potential causative role in the progression of neurodegenerative diseases require further investigation. We aim to (1) assess rotenone-induced alterations to the ALP that contribute to neurodegenerative pathology; (2) characterize ALP function and neuronal toxicity after genetic inhibition ATP6V0C under basal and stressed conditions; and (3) further understand low-dose BafA1-mediated neuroprotection in models of neurodegeneration.

Using an \textit{in vitro} model of PD, we treated differentiated neuronal cells with the environmental pesticide rotenone, a known neurotoxin linked to PD. Although previous studies report rotenone cause autophagosome accumulation, whether this accumulation is due to increased autophagosome production or decreased degradation is not entirely clear. In our studies we observed that rotenone induced accumulation of autophagosomes and $\alpha$-synuclein that resulted from decreased lysosome-mediated degradation. We provide evidence that rotenone-induced lysosome dysfunction is caused by an elevation of pH concomitant with a reduction in cellular energy levels prior to cell death. These studies conclude that rotenone has a pronounced effect on macroautophagy completion that may contribute to its neurotoxic potential.

Lysosome dysfunction is linked to neuronal cell death in many neurodegenerative diseases. As V-ATPase is a critical regulator of lysosome function, we investigated whether knockdown of the V-ATPase subunit, ATP6V0C, could alter the ALP and cause PD-like cellular pathologies. We found that knockdown of ATP6V0C alone caused an elevation in acidic vesicle pH, increased autophagosome accumulation, and decreased autophagic turnover. These observed alterations in the ALP occurred concomitantly with increased detection of $\alpha$-synuclein. Knockdown of ATP6V0C alone caused a decrease in neurite length which was further exacerbated with BafA1 treatment. Although ATP6V0C knockdown alone did not alter cell
viability, it caused a shift in the dose-response sensitizing cells to BafA1 toxicity. Together these results indicate a role for ATP6V0C in maintaining constitutive and stress-induced ALP function, in particular the metabolism of substrates that accumulate in age-related neurodegenerative disease and may contribute to disease pathogenesis.

Our lab has previously shown low concentrations of BafA1 that do not inhibit V-ATPase effectively attenuate ALP-related neuronal pathology by enhancing ALP function in \textit{in vitro} models of neurodegeneration. Additionally, low-dose BafA1 can protect dopaminergic cells from \(\alpha\)-synuclein toxicity. However, prior to these studies whether low-dose BafA1 provided ALP-dependent protection was not known. Using the lysosome inhibitor chloroquine to induce ALP dysfunction and cell death, we investigated whether low-dose BafA1 protection could be maintained in the absence of important ALP-related proteins. siRNA-mediated knockdown of both ATP6V0C and the autophagy related protein Atg7 prevented the BafA1-mediated attenuation of neuronal cell death induced by chloroquine, which induces cell death due to lysosome dysfunction. We also provide evidence that low-dose BafA1 enhances autophagic flux during periods of lysosome dysfunction, an effect that is inhibited by knockdown of ATP6V0C. Our findings suggest that low-dose BafA1 confers neuroprotection directly through enhanced ALP function and provides support for the therapeutic potential of methods which may enhance autophagy to treat neurodegenerative pathologies.

Together, our findings highlight the importance of the ALP in maintenance of neuronal function. As such the ALP may be a useful therapeutic target to attenuate or prevent neuron loss in neurodegenerative disease. Current strategies that are intended to maintain neuronal cell viability and in turn dopaminergic signaling by may prove to be extremely useful in slowing or halting disease progression either alone or in combination with current therapeutic methods.
DEDICATION

I dedicate this work to my daughter Charlotte Sophia Mader whose very existence has inspired and motivated me to persevere through my own perceived limitations. I would like to also dedicate this work to my partner Christa Lyle whose patience, support, and sacrifice have allowed me to pursue all of my interests and passions.
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# INTRODUCTION

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# ATP6V0C KNOCKDOWN IN NEUROBLASTOMA CELLS ALTERS AUTOPHAGY-LYSOSOME PATHWAY FUNCTION AND METABOLISM OF PROTEINS THAT ACCUMULATE IN NEURODEGENERATIVE DISEASE

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INTRODUCTION

Neurodegenerative Disease

Age-related neurodegenerative diseases such as Parkinson’s disease (PD) are among the most devastating and widespread disorders facing our elderly population. The financial toll associated with PD in the United States alone is estimated to cost in the tens of billions of dollars annually in addition to the immeasurable emotional toll taken on the patients and their families. Symptoms associated with neurodegenerative disease diseases result from the dysfunction and/or death of neuronal cells that are critical to maintaining normal sensory, movement, and/or cognitive function. These symptoms range from memory loss to inhibition of neuromuscular control creating a great reduction in a patient’s quality of life. It is therefore of the utmost importance to determine the mechanisms that lead to neuronal dysfunction and/or death so that targeted therapies may be developed to combat neurodegenerative disease.

A common characteristic of age-related neurodegenerative diseases is the brain region-specific accumulation of cellular debris and toxic protein species incorporated into large aggregates. Current research suggests that altered regulation of autophagy, a cellular phenomenon where the cell degrades and recycles its intracellular material to meet its energetic and nutritional needs, may play an important role in the pathogenesis
of several neurodegenerative diseases\textsuperscript{58}. Under normal conditions within neuronal cells the presence of autophagic vesicles, termed autophagosomes, are difficult to detect experimentally as they are efficiently degraded by the lysosome\textsuperscript{58}. However, many neurodegenerative diseases exhibit robust accumulation of autophagosomes, suggesting alterations in autophagy may contribute to neuronal dysfunction and death\textsuperscript{58}. While dysfunctional autophagy is generally accepted as a central participant in pathologies associated with various neurodegenerative diseases, the precise mechanisms which ultimately lead to disease currently remain unclear. It is the goal of our research to identify potential therapeutic methods that reduce the deleterious effects of neurodegenerative disease through the identification of alterations in autophagy which contribute to neurodegeneration.

**Parkinson’s Disease**

PD affects upwards of 1–2\% of persons age 60 or older and is characterized by neuromuscular abnormalities at a functional level and pathologically by the loss of substantia nigra dopaminergic neurons and accumulation of termed Lewy bodies, intracellular inclusions that contain filamentous aggregates of the protein α-synuclein as their major component\textsuperscript{77}. Symptoms from patients suffering from PD include resting tremor (shaking), muscle rigidity, bradykinesia (slow movement), and postural instability. PD represents a class of neurodegenerative diseases called “synucleinopathies,” and are defined by the brain region-specific accumulation of α-synuclein-containing Lewy bodies. It is believed that the loss of substantia nigra
dopaminergic neurons leads to a profound deficit in levels of striatal dopamine required for normal motor function\textsuperscript{25}.

In 1997, PD was first linked to a mutation in SNCA, the gene that codes for α-synuclein, by the substitution of one amino acid\textsuperscript{64}. Subsequently, there have been numerous identified mutations and duplications of SNCA that are directly linked to the accumulation of α-synuclein and PD pathology\textsuperscript{65,73}. These genetic findings have paved the way for the development of refined biological tools such as α-synuclein-specific antibodies to accelerate the study of α-synuclein in PD. Lewy body formation is directly linked to an overabundance of α-synuclein in PD brain. α-Synuclein accumulation is dependent on its gene expression and protein biosynthesis in combination with efficient removal by an intracellular degradation mechanism\textsuperscript{78}. Histological analysis of autopsied PD brains indicate that α-synuclein pathology is widespread and may even follow a progressive migration pattern termed “Braak staging,” which describes detection of α-synuclein pathology that in the brain begins within the olfactory bulb and ends with late stage α-synuclein detection within the cortex\textsuperscript{12}. A recent \textit{in vivo} study determined that the viral over-expression of α-synuclein can cause a decrease in dopaminergic neurotransmission coupled with neuritic swellings containing α-synuclein, both of which are indicative of synaptic dysfunction\textsuperscript{51}. While it is clear that α-synuclein plays a major pathogenic role in the progression of PD, the specific mechanisms by which it causes pathology remain largely unknown. Inhibition of cellular bioenergetics pathways that are critically regulated by the mitochondria have been shown to induce neurodegeneration\textsuperscript{82}. A decreased function of complex I is reported in the substantia nigra of PD patients that may lead to the generation of oxidative stress and a decrease in ATP production\textsuperscript{57}. 3
Furthermore, a study has reported detecting small proportions of α-synuclein within the mitochondria that may down-regulate the activity of the mitochondrial electron transport chain complex I \(^22\), suggesting a potential causal role for α-synuclein and mitochondrial dysfunction in PD.

Widely used to model PD pathology, the drug rotenone is an environmental pesticide that has been shown to induce oxidative stress, α-synuclein accumulation, and dopaminergic neuron death through its selective pharmacological inhibition of mitochondrial complex I \(^7\). Furthermore, exposure to environmental pesticides such as rotenone is a known risk factor for PD\(^7\). Rotenone has also been shown in several studies to cause accumulation of autophagosomes as a likely response to inhibition of mitochondrial function and generation of oxidative stress\(^14,50,52,81,90,101\). However at this time, whether rotenone-induced accumulation of autophagosomes results from either increased induction of macroautophagy or inhibition of lysosomal degradation is under debate.

**The Autophagy-Lysosome Pathway (ALP)**

Autophagy, literally translated from Greek meaning “self-eating,” is an intracellular lysosomal degradation pathway that breaks down cytoplasmic contents including damaged proteins and organelles, and recycles their components for cellular reuse. Christian de Duve first used the term “autophagy” in 1963 in his efforts to establish nomenclature as a means to characterize a variety of cellular trafficking pathways\(^28,40\). Although much of the molecular research leading to the identification of specific autophagy genes (ATG) was conducted in yeast, ATG homologs have been
found to be conserved in higher eukaryotes highlighting the importance of autophagy from an evolutionary perspective\textsuperscript{33}. These ATG genes code for numerous proteins (Atg) involved in the regulation of the ALP and it is widely accepted that dysregulation of these autophagic proteins can promote neuronal toxicity by causing deleterious effects on autophagic processes.

Neurons are post-mitotic and as such they rely heavily upon the ALP to maintain energy balance and organellar quality control without the putative protective effects of cell division. The intracellular quality control that the ALP provides a neuron is essential for its survival. Depletion of lysosome number is a prevalent finding in the aged brain and may have a great impact on the brain’s ability to maintain a healthy intracellular environment\textsuperscript{19}. This depletion of lysosomes (and implied compromise in effective autophagy) may account for elevated levels of putatively toxic proteins such as amyloid beta and tau in AD, and \(\alpha\)-synuclein in PD. In the past decade various signaling pathways and modulators of the ALP have been proposed in numerous studies as promising targets for the discovery of therapies to combat many various neurodegenerative diseases\textsuperscript{15}.

Based on the cellular environment and nutrient levels within the cell at any given time, autophagy can be classified as either “basal” (relatively low-level demand for autophagy) or “stress-induced” (high level)\textsuperscript{16,33}. Basal, or constitutive autophagy, serves as an essential intracellular quality control mechanism by removing aggregation-prone proteins and damaged organelles and in turn maintains energy balance under normal conditions. During periods of stress, autophagy can be upregulated to increase the turnover of intracellular material to meet the cell’s metabolic needs. Autophagy provides cells with the ability to adapt to environmental stresses and is thus considered to directly
contribute to maintenance of cellular viability\textsuperscript{15}. Although there are recent descriptions of very specific subsets of autophagy the ALP can be divided into three principal types based on how the cargo is transported to the lysosome: microautophagy, chaperone-mediated autophagy, and macroautophagy.

Microautophagy is the process of pinocytosis, the direct uptake of defined small volumes of cytosol by the lysosome. This autophagic process happens without the participation of vesicles or chaperone proteins as the lysosome appears to invaginate or project arm-like protrusions to sequester the cytosolic constituents that are proximal to the lysosome\textsuperscript{56}. Microautophagy in mammalian cells has been described but the majority of information learned about this pathway has resulted from studies of yeast\textsuperscript{67, 85}.

Chaperone-mediated autophagy is considered to be highly selective in its degradation of cytosolic proteins. Chaperone-mediated autophagy degrades proteins containing a KFERQ amino acid motif, which is recognized by the 70kDa cytosolic chaperone heat shock protein, Hsc70. Subsequently the chaperone and its cargo are transported to the lysosome where lysosome associated membrane protein type 2a (LAMP-2a) mediates intraluminal substrate translocation for degradation. Diminished levels of both LAMP-2a and Hsc70 have been previously documented in PD brain\textsuperscript{2}. Chaperone-mediated autophagy activity has been shown to decline with age in a variety of tissues and is attributed to depleted LAMP-2a levels. Chaperone-mediated autophagy has been reported in many studies to regulate the degradation of $\alpha$-synuclein\textsuperscript{53, 84, 91-93}. Furthermore, there is strong evidence that chaperone-mediated autophagy and macroautophagy are closely interconnected in the process of $\alpha$-synuclein degradation and that blockage of one of these pathways causes an upregulation of the other\textsuperscript{54, 92}.
The primary focus of our research is macroautophagy and its regulation is explained in detail below, however the abbreviated description of the other types of autophagy does not intend to diminish the impact chaperone-mediated autophagy, microautophagy, and other specialized types of autophagy may have on cellular function and survival.

**Macroautophagy**

Macroautophagy is a subset of autophagy that regulates intracellular degradation by sequestering cytosolic material within double-membraned vesicles called autophagosomes. Autophagosomes were first observed by electron microscopy in the 1950s, however most of what we have learned about macroautophagy has been published within the past 15 years. These autophagosomes are transported along the microtubule network from other parts of the cell by dynein ATP motors to perinculear lysosomes where acidic hydrolases degrade the sequestered cellular material and subsequently release their components for reuse.

The initial phase of macroautophagy is termed “induction.” Nutrient deprivation (mTOR-dependent), oxidative stress, and infection are stimuli known signal the increased formation of autophagosomes. This induction phase leads into the maturation and expansion phase.

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**Fig. 1. Illustrated Macroautophagy Pathway**
with the formation of a cup-shaped membrane within the cytoplasm and is called a phagophore (Fig. 1). While the origin of the phagophore remains unclear, recent studies have suggested it may initiate from the endoplasmic reticulum, golgi, mitochondria, or plasma membrane. After phagophore formation multiple protein-protein and protein-lipid conjugation reactions continue to occur. As the membrane elongates and finally completes its expansion the double-membraned autophagosomes sequesters cytoplasmic cargo destined for degradation.

The efficient regulation of autophagosome formation is maintained by two interrelated ubiquitin-like conjugation systems that form the phagophore. The complexes formed by these conjugation systems, Atg12-Atg5-Atg16 and LC3-PE (microtubule-associated protein-light-chain-3-II-phosphatidylethanolamine), are essential for phagophore maturation and autophagosome formation. Atg12 is activated by Atg7 (activating enzyme) and subsequently transported to Atg10 (a conjugating enzyme) where Atg12 is covalently conjugated to Atg5. This Atg12-Atg5 duplex is conjugated further with Atg16 and through a self-oligomerization reaction the Atg12-Atg5-Atg16 conjugate forms a tetramer and attaches itself to the expanding phagophore.

Simultaneously, LC3 is initially processed by the protease Atg4 and is activated by the same Atg7 from the Atg12-Atg5-Atg16 pathway. Next, this modulated LC3 protein is conjugated to the target lipid, PE, converting it from the cytosolic LC3-I into the membrane bound LC3-II that is incorporated into the growing phagophore until the autophagosome is finally formed. Several ALP-associated molecules have been identified that are currently being investigated for their utility as therapeutic targets in age-related neurodegenerative disease. One study of particular interest demonstrated
PD-like symptoms in mice and was linked to deficiency of Atg7, including age-related dopaminergic neuron loss concomitant with α-synuclein accumulation\(^1\), suggesting autophagy is important for the degradation of putatively toxic proteins species.

Following complete formation of the autophagosome, the next step in the macroautophagy pathway requires the hydrolysis of ATP to drive dynein motor-transport of autophagosomes along microtubule networks to deliver their cargo to the lysosome for degradation\(^38,45\). Transport of these autophagosomes occurs from distal regions of the cell towards the perinuclear region where lysosomes are located. Alterations in dynein that affect transport are reported with the accumulation of autophagosomes and ALP substrates associated with disease pathology seen in PD, Alzheimer’s disease and other neurodegenerative diseases\(^27\). While direct links between dynein and neurodegeneration are limited, there are a number of mutant genes that regulate dynein expression that are found in PD and other neurodegenerative disease\(^27\).

Once delivered to lysosomes, autophagosomes tether, dock and then fuse with lysosomal membranes in separately regulated events, independent of lysosomal acidification\(^55\). Once fusion is complete, the autolysosome is formed and the autophagic cargo will be degraded by lysosomal hydrolases. There is evidence that membrane-associated proteins such as the LAMP family and V-ATPase proteins may regulate these steps leading to partial or complete fusion of the autophagosome to acidic vesicles\(^36,42\). The mechanisms by which fusion is regulated by these lysosome membrane proteins currently remain unclear.

Acidic hydrolases are essential for substrate degradation and reduction of their functional activity has been shown to contribute to many disease processes. Pathology
can be caused by either genetic mutation in lysosomal storage diseases \(^7\) that lead to robust substrate accumulation, or environmental stressors (inhibition of hydrolases or de-acidification of the lysosome) that alter lysosome-mediated degradation. The products of degradation are then sent back out into the cytosol for cellular reuse.

Although the ALP is believed to provide a pro-survival function at the cellular level, dysfunction of the ALP may also potentially contribute to pathologies observed concomitant with an overabundance of autophagosomes. Increased detection of autophagosomes is found in several human neurodegenerative diseases including AD, PD, and dementia with Lewy bodies \(^4, 20, 59\).

While it remains controversial as to whether the accumulation of autophagosomes indicates a causal role of the ALP in neuronal cell death, dysfunctional degradation through inhibition of ALP function is widely considered a key player in the pathology of age-related neurodegenerative disease. The ALP is described as the main degradation pathway for \(\alpha\)-syn oligomers and aggregates due to its large capacity when compared with the proteosome, further implicating the potential for ALP dysfunction in PD pathology \(^{47, 84, 93}\). Therefore, it is important to elucidate alterations in the ALP that contribute to disease pathogenesis, giving special consideration to \(\alpha\)-synuclein in PD and metabolites of amyloid precursor protein in Alzheimer’s disease.

**\(\alpha\)-Synuclein Degradation**

\(\alpha\)-Synuclein is a 140 amino acid natively unfolded protein and in the nervous system accounts for approximately 1% of cytosolic protein. \(\alpha\)-Synuclein is detected in the early stages of neuronal development when synapses are beginning to form. Recent
studies propose α-synuclein may regulate synaptic neurotransmission through its association with the vesicles that release neurotransmitters. The accumulation of α-synuclein, a presynaptic neuronal protein genetically linked to PD neuropathology seems to occur through a combination of independent or interrelated factors including increased expression, genetic mutation, post-translational modification, and decreased clearance by degradation pathways.

The ubiquitin-proteasome system and the ALP are considered the two main routes by which α-synuclein is degraded. It is reported that the UPS is primarily responsible for degrading the α-synuclein monomer, while the ALP’s larger size capacity allows for the degradation of the higher molecular weight species of α-synuclein suspected of causing neuronal toxicity. α-Synuclein degradation becomes even more complicated under experimental conditions as it can inhibit both the ubiquitin-proteasome system and the ALP, creating an environment that is primed for the emergence of a toxic feedback loop in aberrant α-synuclein accumulation. Although these degradation pathways have been mostly accepted as the primary methods by which α-synuclein is degraded, the specific mechanisms that regulate its degradation are still in question.

After experimentally inducing lysosome dysfunction, α-synuclein is reported to be secreted in association with exosomes that may exert toxic potential upon neighboring cells. Induction of paracrine neurotoxicity toxicity is exacerbated by dysfunction of autophagy. These studies highlight the importance of understanding the relationship of the ALP to α-synuclein metabolism so that therapies can be developed to enhance clearance of α-synuclein in a way that reduces neurotoxicity.
Lysosome Dysfunction

Lysosome dysfunction leads to a reduced capacity for cellular degradation triggering the accumulation of cellular debris and large inclusions of aggregated proteins. Acidiotropic drugs such as chloroquine are used experimentally to characterize lysosome dysfunction. Chloroquine is a weak base that is attracted to acidic environments and can move freely across the lysosome membrane. However, upon arrival in the lysosomal lumen chloroquine molecules become trapped by the addition of a proton leading to its intralysosomal accumulation and resultant elevation of lysosomal pH.$^{74}$ Inhibition of the lysosomal enzymes that are dependent on acidic pH for optimal function can occur due to increased lysosome pH. We have previously published data showing chloroquine causes cell death and inhibition of autophagy with accumulation of proteins with neurotoxic potential.$^{60, 72}$

The Lysosome and V-ATPase

Drugs such as chloroquine and bafilomycin A1 (BafA1) are known to induce pH-dependent lysosome dysfunction through a variety of different mechanisms.

BafA1 is categorized in the plecomacrolide subclass of macrolide antibiotics and is reported to selectively inhibit vacuolar type-ATPase (V-ATPase, Fig. 2)$^{11}$.

Inhibition of V-ATPase is shown to be mediated by the binding of BafA1 with high affinity to the c subunit of its $V_0$ domain, or ATP6V0C.$^{9, 10}$ Unlike chloroquine, BafA1 causes lysosome dysfunction through its
inhibition of the V-ATPase pump, an essential component of lysosomal membrane machinery that regulates proton movement to maintain lysosome pH.

At concentrations ≥10 nM, BafA1 inhibits V-ATPase and in turn increases intravesicular pH, thus mimicking the effects of chloroquine. Experimental inhibition of V-ATPase by BafA1 has been shown to prevent the effective degradation of α-synuclein and in turn promote the accumulation of α-synuclein associated with neurotoxic potential.

As pharmacological inhibition of V-ATPase has been shown experimentally to promote α-synuclein accumulation, further targeting this enzyme complex may provide greater information regarding its role in regulating the basal vs. stress-induced metabolism of α-synuclein. V-ATPase is a membrane-associated, multi-subunit protein complex that functions as an ATP-driven proton-pump and is localized to many different membranes of eukaryotic cells including lysosomes, endosomes, Golgi-derived vesicles, secretory vesicles and in some cell types the plasma membrane. V-ATPase maintains the low pH of acidic vesicles through its regulation of proton pumping.

The V-ATPase is a large complex that is comprised of two domains: the V1 domain, which can be found either associated with the full complex or disassociated within the cytoplasm and is responsible for ATP hydrolysis; and the V0 domain, which putatively rotates like a wheel to pump protons across the membrane into the lumen of the lysosome. Any cellular stress that impedes the ability of V-ATPase to maintain the internal lysosomal pH may potentially harm the ALP’s degradative capacity thus increasing the likelihood of cellular dysfunction and death.
Knockdown of ATP6V0C, a subunit of the V0 domain, has been shown recently to inhibit vesicular acidification and sensitize cells to stress-induced cell death\(^{13}\). Alternatively, over-expression of ATP6V0C has been shown to contribute to the rescue of dopaminergic signaling in a model of PD\(^{39}\). Whether ATP6V0C itself is responsible for regulating ALP function or the metabolism of substrates that accumulate in age-related neurodegenerative diseases has not been previously investigated.

**Low-dose Bafilomycin-mediated Neuroprotection**

Our lab has demonstrated that the plecomacroldes BafA1, bafilomycin B1 and concanamycin, in addition to their known inhibition of V-ATPase, all significantly attenuate chloroquine-induced death of cerebellar granule neurons\(^{71,72}\) at low concentrations (≤ 1 nM) which do not inhibit V-ATPase\(^{11}\) or induce autophagosome accumulation\(^{72}\). These data suggest that low-dose BafA1-mediated neuroprotection is independent of its inhibition of V-ATPase. Subsequent studies in different cell lines have exhibited BafA1-mediated neuroprotection using concentrations of BafA1 that do not alter neuronal viability or acidic vesicle pH\(^{60}\). Low-dose BafA1 treatment also is shown to attenuate α-synuclein oligomer accumulation that is triggered by chloroquine exposure by some mechanism that attenuates the inhibition of autophagic flux\(^{60}\). Finally, BafA1-mediated neuroprotection was confirmed through its inhibition of α-synuclein-associated toxicity in *C. elegans*, a worm model used to study PD pathologies in dopaminergic neurons\(^{60}\).
While these studies provide strong evidence of BafA1-mediated neuroprotection, the mechanisms by which low-dose BafA1 affects the ALP or regulates α-synuclein clearance and neurotoxicity has not been investigated.

**Aims of this Dissertation**

The ALP and V-ATPase represent attractive targets for promoting the metabolism and turnover of proteins that contribute to the pathogenesis of age-related neurodegenerative disease such as α-synuclein in PD. The aims of this dissertation are (1) assess rotenone-induced alterations to the ALP that contribute to neurodegenerative pathology; (2) characterize ALP function and its contribution to neuronal toxicity after genetic inhibition of the V-ATPase subunit ATP6V0C under basal and stressed conditions; and (3) elucidate the mechanism that regulates low-dose BafA1-mediated neuroprotection from pharmacologically-induced lysosome dysfunction.

Although it is currently accepted that alterations in the ALP contribute to neurodegenerative disease there remains very little known about how this pathway directly impacts cell survival within the human brain and causes disease. These studies are intended to identify the dysfunctional mechanisms that may potentially occur in the brains of PD patients or other patients diagnosed with neurodegenerative disease. The ALP appears to be an important homeostatic mechanism in neuronal cells under normal conditions, however under periods of stress the ALP’s role has been shown to provide protection as well as to contribute to toxicity.
ROTHENONE INHIBITS AUTOPHAGIC FLUX PRIOR TO INDUCING CELL DEATH

by

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ABSTRACT

Rotenone, which selectively inhibits mitochondrial complex I, induces oxidative stress, α-synuclein accumulation and dopaminergic neuron death, principal pathological features of Parkinson disease. The autophagy-lysosome pathway degrades damaged proteins and organelles for the intracellular maintenance of nutrient and energy balance. While it is known that rotenone causes autophagic vacuole accumulation, the mechanism by which this effect occurs has not been thoroughly investigated. Treatment of differentiated SH-SY5Y cells with rotenone (10 μM) induced the accumulation of autophagic vacuoles at 6h and 24h as indicated by western blot analysis for microtubule associated protein-light chain 3-II (MAP-LC3-II). Assessment of autophagic flux at these time points indicated that autophagic vacuole accumulation resulted from a decrease in their effective lysosomal degradation, which was substantiated by increased levels of autophagy substrates p62 and α-synuclein. Inhibition of lysosomal degradation may be explained by the observed decrease in cellular ATP levels, which in turn may have caused the observed concomitant increase in acidic vesicle pH. The early (6h) effects of rotenone on cellular energetics and autophagy-lysosome pathway function preceded the induction of cell death and apoptosis. These findings indicate that the classical mitochondrial toxin rotenone has a pronounced effect on macroautophagy completion that may contribute to its neurotoxic potential.
INTRODUCTION

Parkinson disease (PD) is an age-related neurodegenerative disorder that affects upwards of 1-2% of persons age 60 or older and is characterized pathologically by the loss of substantia nigra dopaminergic neurons and accumulation of intracellular protein inclusions termed Lewy bodies\(^1\). Oxidative stress and a decreased capacity of neurons to degrade \(\alpha\)-synuclein, a protein reportedly involved in vesicular dopamine release and the major component of Lewy bodies, are believed to be major contributors to Lewy body formation and neurodegeneration in PD\(^2\).

A decreased function of complex I of the mitochondrial electron transport chain is reported in the substantia nigra of PD patients\(^1\) that may lead to the generation of oxidative stress and a decrease in ATP production. One mechanism by which cells compensate for this disruption in energy balance is through stimulation of the autophagy-lysosome pathway (ALP), which shuttles outlived and/or damaged proteins and organelles to the lysosome for their pH-dependent degradation and recycling\(^3\)\(^-\)\(^5\). In macroautophagy, a subset of the ALP, double-membraned autophagic vacuoles shuttle cargo destined for degradation to lysosomes. Increased numbers of autophagic vacuoles are detected in several human Lewy body diseases including PD and dementia with Lewy bodies\(^6\)\(^-\)\(^8\) as well as in animal models of PD\(^9\). Moreover, the ALP is believed to be the main route for degradation of \(\alpha\)-synuclein oligomers and aggregates, further implicating its dysfunction in PD pathology\(^10\).

Exposure to environmental pesticides such as rotenone, a selective inhibitor of complex I of the mitochondrial electron transport chain, is associated with an elevated risk of developing PD and is used experimentally to model PD pathophysiology\(^11\)\(^,\)\(^12\). Rotenone induces neurodegeneration and neuron death in association with caspase activation\(^13\), and causes \(\alpha\)-
synuclein accumulation in experimental models of PD. Moreover, rotenone-induced oxidative stress reportedly induces post-translational modifications of α-synuclein, which are thought to promote its aggregation and toxicity.

Rotenone has also been shown in several studies to cause accumulation of autophagic vacuoles, as a likely response to inhibition of mitochondrial function and generation of oxidative stress. However, it is not entirely clear if rotenone-induced accumulation of autophagic vacuoles results from either an increased induction of macroautophagy or from the inhibition of macroautophagy completion, which requires functional fusion of autophagic vacuoles with lysosomes. Such information may be important for designing rational therapeutics that attenuate PD-associated neuronal dysfunction, in part through their maintenance of ALP function. Thus the goal of this study was to delineate the mechanism by which autophagic vacuoles accumulate in cultured neuronal cells following treatment with an acute, death-inducing concentration of rotenone.

**RESULTS AND DISCUSSION**

It is well established that rotenone disrupts the ALP. However, the cause of autophagic vacuole accumulation following rotenone treatment has not been carefully investigated. The purpose of this study was to assess the manner by which rotenone, a potent mitochondrial toxin that is used as an experimental model of PD, affects accumulation of autophagic vacuoles using a neuroblastoma cell line (SH-SY5Y) that was differentiated to a neuronal phenotype. We first assessed cell death, apoptosis and energetics following treatment
with rotenone. Next, using a death-inducing concentration of rotenone we assessed autophagic flux to determine the manner by which autophagic vacuoles accumulate. We next assessed vesicular acidification and the integrity of lysosomal membranes to determine the potential for acidic vesicle dysfunction to contribute to changes observed in autophagic flux. Finally, we correlated these observed effects of rotenone on macroautophagy by assessing relative levels of a lysosomal membrane marker and a transcription factor that is responsible for its stress-induced up-regulation.

**Rotenone-induced neuronal cell death and apoptosis.**

In retinoic acid-differentiated SH-SY5Y cells, rotenone induced concentration and time-dependent cell death (Fig. 1A, C). A decrease in cell viability was observed beginning at 24h of 10µM rotenone treatment. About a 50% decrease in SH-SY5Y cell viability was observed after 48h of rotenone treatment, which progressed to 70% by 72h (Fig. 1C). Rotenone-induced SH-SY5Y cell death was accompanied by a concentration-dependent increase in caspase 3-like activity (Fig. 1B). A three to four-fold increase was also detected in caspase 3-like enzymatic activity in 10µM rotenone treated SH-SY5Y cells vs. vehicle control at 48 and 72h, indicating that rotenone-induced cell death was accompanied by caspase activation (Fig 1D). All subsequent experiments utilized a concentration of 10 µM rotenone. To determine if caspase activation was required for rotenone-induced neuron death, we measured cell viability in the presence of a broad caspase inhibitor Boc-Asp(OMe)-FMK (Boc-FMK) (Fig 1E). Boc-FMK at a concentration that completely inhibited rotenone-induced caspase-3-like activity (data not shown) failed to inhibit rotenone-induced cell death. This data indicates that although rotenone can induce robust apoptosis, caspase activation per se, is not required for cell death. To confirm
that a death-inducing concentration of rotenone affected cellular energetic, ATP levels were measured at 6h and 12h following rotenone treatment (Fig 1F), time points that preceded the induction of neuronal apoptosis and cell death (Figs 1C-1D). Rotenone caused a significant reduction in ATP levels at both time points, due likely to the inhibition of complex I of the mitochondrial electron transport chain and indicates the reliance of these cells on mitochondrial ATP production as a source of energy.

**Rotenone causes early and persistent accumulation of autophagic vacuoles by compromising their lysosomal degradation.**

Previous studies have identified accumulation of autophagic vacuoles in substantia nigra neurons of PD brain and in *in vitro* models of rotenone-induced neuronal cell death, suggesting that the ALP is involved in regulating dopaminergic neuron death. To further delineate the involvement of macroautophagy in rotenone-induced neuronal cell death, we assessed levels of autophagic vacuoles via western blot analysis for MAP-light chain 3-II (LC3-II; Fig. 2), an accepted and selective marker of autophagic vacuoles. Treatment with rotenone induced a significant accumulation of autophagic vacuoles at both 6h (Fig. 2B) and 24h (Fig. 2C) after treatment. Since the accumulation of autophagic vacuoles may result from either induction of autophagy or from a decrease in their lysosomal degradation, we also assessed autophagic flux (Fig. 2) via treatment with 100 nM bafilomycin A1, a selective inhibitor of vacuolar-type V-ATPase that completely blocks degradation via macroautophagy through its inhibition of autophagic vacuole-lysosome fusion. Bafilomycin A1 was added to cells at 4h or 22h (2h prior to collection at the 6h and 24h rotenone time points, respectively). At each time point tested, we found no further increase in levels of LC3-II in bafilomycin A1-rotenone-treated cells compared
to cells treated only with 100nM bafilomycin A1 (Figs. 2B-2C). This result suggests that the rotenone-induced increase in autophagic vacuoles resulted not from autophagy induction but rather from a block in the lysosomal degradation of autophagic vacuoles. As a positive control for autophagy induction, the addition of 100 nM BafA1 for the last 2h of a 24h rapamycin treatment produced noticeably greater LC3-II immunoreactivity in comparison to 2h treatment with BafA1 alone (data not shown).

Autophagic flux is also commonly assessed by detecting protein levels of p62/A170/SQSMT1, a ubiquinating and LC3 binding protein that binds to ubiquitin aggregates and promotes degradation via autophagy\textsuperscript{22}. Thus decreases in p62 levels are associated with enhanced autophagic flux as observed for example during serum starvation, whereas its accumulation suggests autophagy degradation block\textsuperscript{22}. We observed a significant increase in p62 levels at both 6h and 24h following treatment with 10 µM rotenone (Figs. 2D-E). These data support our assessment of autophagic flux (Figs. 2A-C) suggesting that rotenone disrupts the degradation of autophagic vacuoles. Further evidence for rotenone-induced inhibition of autophagic degradation in our model was supported by a significant increase in high-molecular weight species of alpha synuclein at 48h following treatment (Fig. 3), as it is well established that the ALP is important for α-syn degradation\textsuperscript{8, 23, 24}. Levels of alpha synuclein following 6h and 24h of rotenone treatment were not significantly different from vehicle control (data not shown).

\textbf{Rotenone increases acidic vesicle pH but does not induce lysosomal membrane permeabilization.}

To determine if rotenone-induced inhibition of autophagic flux correlated with a compromise in the function of acidic vesicles, we utilized a flow cytometric approach with the
acidotropic dye lysotracker red (LTR) to quantify acidic vesicle pH (Fig. 4). Rotenone induced a significant, >30% loss in LTR mean fluorescence intensity vs. vehicle control-treated cells at both 6h and 24h following treatment, time points that coincided with the observed decrease in ATP production (6h; Fig. 1G) and inhibition of macroautophagy (Fig. 2). The effects of rotenone on LTR mean fluorescence intensity were not as robust as those observed following treatment with 100 nM BafA1, a potent inhibitor of acidic vesicle pH via its direct inhibition of V-ATPase (refs). Epifluorescence microscopy images (Fig. 4C) corroborate our findings via flow cytometry, where compared to vehicle control-treated cells there is a relative lack of LTR fluorescence following treatment with rotenone or bafilomycin A1. These results indicate that rotenone causes an early and persistent increase in acidic vesicle pH, which may be due possibly to a net decrease in cellular ATP levels and in turn ATP-dependent acidification. Rotenone has been shown previously to inhibit cathepsin D activity and decrease fluorescence emitted by the acidotropic dye lysosensor green in cultured cell lines25,26, evidence that corroborates our findings.

We also determined if rotenone caused the onset of lysosomal membrane permeabilization (LMP) by assessing the co-localization of cathepsin D and LAMP-1 immunoreactivity using confocal microscopy (Fig. 5). At 24h after treatment with rotenone, a time point that corresponded to significant inhibition of autophagic flux (Fig. 2), a significant decrease in LTR mean fluorescence intensity (Fig. 4) and the onset of cell death/apoptosis (Fig. 1), cathepsin D immunoreactivity co-localized with LAMP-1 similar to that of vehicle control-treated cells, suggesting a lack of LMP at this time point. In contrast, noticeable LMP (as indicated by a diffuse staining pattern for cathepsin D that did not co-localize with LAMP-1) was observed in cells treated for 24h with chloroquine (50 µM; Fig. 5), a lysosomotropic agent and
known inducer of LMP\textsuperscript{27}. Together these results suggest that the inhibition of autophagic flux by rotenone may be caused in part by its inhibition of vesicular acidification, perhaps via its potent inhibition of intracellular ATP, but not because of the onset of LMP. It is possible that rotenone may eventually induce LMP at later time points not tested in this study, as shown previously by the dopaminergic neurotoxin MPP\textsuperscript{4}\textsuperscript{9} and the oxidative stress-inducing agent hydrogen peroxide\textsuperscript{28}. However, the possibility still exists that rotenone-induced oxidative stress in our model may compromise lysosomal membrane integrity that in turn may decrease the efficiency of macroautophagy for clearing substrates\textsuperscript{5}, but at a lower concentration and earlier time than that is required for inducing LMP. Taken together, lysosome dysfunction corresponds with and may contribute to a decreased turnover of autophagic vacuoles following rotenone treatment, events that may be responsible for the accumulation of macroautophagy substrate and induction of cell death.

**Rotenone increased levels of LAMP-1 but not TFEB.**

We also tested the effect of rotenone on levels of lysosomal membrane associated protein 1 (LAMP-1), a structural protein present on membranes of late endosomes and lysosomes. LAMP-1 is best known for its regulation of lysosomal motility and endosomal-lysosomal fusion with autophagic vacuoles\textsuperscript{29}, and has been shown to fluctuate with changes in lysosomal volume and/or number\textsuperscript{9,30}. Western blot analysis indicated an increase in LAMP-1 levels following treatment with 10 µM rotenone (Fig. 6A), an effect that was significantly different vs. vehicle control at 6h but not 24h (Fig. 6B). This increase in LAMP-1 suggests either an increase in the number or size of acidic vesicles that may be a consequence of lysosome dysfunction (Fig. 4) and inhibition of autophagic flux (Fig. 2). Conversely, LAMP-1 may also increase as a function of de novo lysosome biogenesis, as shown recently under stressful conditions and as a response
to alterations in autophagy\textsuperscript{9,30}. To address this possibility the effects of rotenone on endogenous levels of the transcription factor EB (TFEB) were assessed by western blot analysis, as TFEB has been shown previously to regulate transcription of LAMP-1 in addition to other lysosomal targets\textsuperscript{9,30}. Levels of TFEB at 6h and 24h following rotenone treatment were similar in rotenone vs. vehicle control cells (Fig. 7), suggesting that the increase in LAMP-1 observed following rotenone treatment does not result from lysosome biogenesis, but rather from the inhibition of lysosomal degradation.

Our assessment of autophagic flux suggests that induction of macroautophagy is not the major mechanism by which rotenone causes accumulation of autophagic vacuoles. In agreement with this interpretation, previous reports have shown that cell death and markers of apoptosis induced by 1-10 µM rotenone in SH-SY5Y are not influenced by siRNA knockdown of Atg5, a gene that is critical for de novo synthesis of autophagic vacuoles\textsuperscript{19,34}. Interestingly, these studies also showed that pre-treatment of SH-SY5Y cells with drugs that induce autophagy attenuate cell death and apoptosis caused by rotenone post-treatment\textsuperscript{19,34}. These observations suggest that, in addition to its ability to block the lysosomal degradation of autophagic vacuoles, rotenone may also inhibit autophagy induction, an effect that is somehow circumvented upon pre-treatment with drugs that stimulate formation of autophagic vacuoles. In support of this hypothesis, it has been shown that rotenone causes de-phosphorylation of death-associated protein kinase (DAPK) in association with mitochondrial dysfunction\textsuperscript{35}. The “active” phosphorylated form of DAPK has been shown to phosphorylate the BH3 domain of Beclin-1, thus disrupting the Bcl-XL-Beclin-1 interaction and in turn promoting Beclin-1-dependent autophagy.

Alternatively, it is possible that rotenone inhibits autophagy induction through its effects on microtubule assembly, as an intact microtubule network is also important for the formation of
*de novo* autophagic vacuoles. Rotenone has been shown previously to inhibit microtubule assembly, as well as increase levels of free tubulin in association with a decrease in mitochondrial membrane potential. An intact microtubule network is known to be important not only for the formation of nascent autophagic vacuoles but also for the fusion of autophagic vacuoles with lysosomes and endosomes. Thus, if rotenone disrupts the function of microtubules, it would not be surprising if autophagic vacuoles accumulate due to an effective block in their trafficking to lysosomes for degradation. Reduced turnover of autophagic vacuoles as a result of microtubule dysfunction may also contribute to persistent rotenone-induced mitochondrial toxicity, as the ability of damaged mitochondria to be effectively recycled by mitophagy (a type of macroautophagy selective for mitochondrial degradation) may be severely compromised.

Other reports indicate that rotenone exhibits concentration-specific or even cell type-specific effects on autophagy induction. Treatment of mouse embryonic fibroblasts with 2 µM rotenone caused accumulation of autophagic vacuoles concomitant with a reduction in levels of p62, suggesting autophagy induction. Concurrent treatment of SH-SY5Y cells with 100 nM rotenone and either rapamycin, which induces autophagy, or 3-MA, which inhibits autophagy induction, attenuated or exacerbated cell death respectively, suggesting that autophagy induction is not inhibited by low concentrations of rotenone. In addition, inhibition of autophagy induction (3-MA vs Atg5 or Beclin-1 siRNA knockdown) in HEK 293 and U87 cells was shown to attenuate cell death induced by 50 µM rotenone, suggesting again that rotenone does not inhibit autophagy induction and that autophagy induction may potentiate rotenone-induced cell death at higher concentrations. We have observed previously using knockdown of Atg7 that autophagy induction contributes to the onset of cell death induced by chloroquine, a
lysosomotropic agent that inhibits autophagic flux\textsuperscript{38}, suggesting that autophagy induction can be death-inducing in the face of lysosome dysfunction.

In conclusion, to our knowledge this is the first report indicating that rotenone inhibits the lysosomal degradation of autophagic vacuoles. Inhibition of autophagic flux was observed at both early and late time points, and may be caused by the rotenone-induced increase in acidic vesicle pH and decrease in ATP levels. The induction of cell death and apoptosis was only apparent at later time points, suggesting that the inhibition of autophagic turnover by rotenone may contribute to cellular demise. In light of the relationship of rotenone as a neurotoxin model of PD, preservation and/or enhancement of autophagic degradation should be considered a viable therapeutic strategy to delay disease onset and/or progression.

METHODS

Cell Culture

SH-SY5Y human neuroblastoma cells were cultured in Minimum Essential Medium Eagle (MEM) (Cellgro, Herndon, VA) and F12-K Nutrient Mixture (ATCC, Manassas, VA) medium supplemented with 0.5% sodium pyruvate, 0.5% non essential amino acids (Cellgro, Herndon, VA), 1% penicillin/streptomycin (Sigma, St. Louis, MO), and 10% Fetal Bovine Serum (FBS) (HyClone, Logan, UT). SH-SY5Y cells were differentiated in 10% FBS feeding media supplemented with 10\textmu M retinoic acid (Sigma, St. Louis, MO) for 7-8 days. Medium supplemented with retinoic acid was replaced every 2-3 days. For experiments, cells were seeded at a density of 400/mm\textsuperscript{2} in media containing 2% B-27 supplement (Invitrogen, Grand Island,
NY) and 10μM retinoic acid. Rotenone (Sigma, St. Louis, MO) stock in DMSO was prepared fresh for every experiment and added to differentiated SH-SY5Y cells for 3-72h.

**Measurement of Cell Viability and Caspase-3-Like Activity**

Cell viability was measured via Calcein AM fluorogenic conversion assay (Invitrogen, Grand Island, NY) and caspase-3-like activity was detected via fluorogenic DEVD cleavage assay and expressed relative to untreated controls as previously described in our laboratory\(^39\).  

**Measurement of ATP Levels**

20,000 differentiated SH-SY5Y cells were plated per well of 96 well plates and after 24h were treated with either DMSO vehicle or rotenone at a final concentration of 10 µM. At 6h and 12h following rotenone treatment levels of ATP were assessed using the ATPLite™ Luminescence ATP Detection Assay System (Perkin Elmer, Waltham, MA). Luminescence was quantified using a BioTek Synergy 2 luminometer. ATP levels (µM/well) were calculated based on a standard curve utilizing an ATP standard supplied by the assay kit. Mean ATP levels were averaged from 6 wells per treatment per time point, and each time point was repeated for a total of 3 independent experiments.

**Western Blot Analysis**

Whole cell lysates were prepared in buffer containing 1% SDS and 1% Triton X-100 as previously described in our laboratory\(^40\). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Equal amounts of protein were electrophoresed on SDS-polyacrylamide gels and subsequently transferred to PVDF membranes (BioRad, Hercules, CA). Western blots were probed for LC3 (Abcam, Cambridge, MA), p62 (Abnova, Littleton, CO),
LAMP1 (1D4B; University of Iowa Hybridoma Bank) or alpha synuclein (Santa Cruz, Santa Cruz, CA). GAPDH (Cell Signaling, Beverly, MA) or actin (Sigma, St. Louis, MO) were used as loading controls. X-ray films of western blots were scanned for densitometric analysis using UN-SCAN-IT gel 6.1 software (UN-SCAN-IT, Orem, UT).

**Assessment of lysosomal membrane permeabilization (LMP)**

To assess LMP, double-label immunocytochemistry was performed for cathepsin-D and LAMP-1. Cells plated in 8-well chamber slides were treated for 24h with either DMSO vehicle, rotenone (10 µM) or chloroquine (50 µM), then were fixed for 20 min with ice-cold 100% methanol. Following PBS wash, fixed cells were incubated for 30 min with blocking buffer (5% v/v horse serum in 1X PBS containing 1% Triton X-100) followed by overnight incubation in blocking buffer without Triton X-100 and containing goat anti-cathepsin D antibody (Santa Cruz, Santa Cruz, CA). After PBS wash, cells were incubated in blocking buffer containing HRP-conjugated anti-goat IgG secondary antibody (ImmPRESS, Vector Laboratories, Burlingame, CA) for 1h in the above blocking buffer, RT followed by PBS wash. Cells were next subjected to a second fixative (4% paraformaldehyde, 15 min, 4°C) for 15 min to prevent membrane rupture that occurs following exposure of methanol-fixed cells to the high salt concentration of our detection reagent buffer. Following PBS wash, detection was performed using Tyramide Signal Amplification (TSA; Perkin Elmer, Waltham, MA) by addition of Cy3-conjugated tyramide in Plus Amp Buffer (30 min, RT) followed by PBS wash. At this point, fixed cells were incubated in blocking buffer (1% BSA, 0.2% evaporated milk, 0.3% Triton X-100 in PBS) for 30 min at RT then overnight in blocking buffer without Triton X-100 and containing with the second primary antibody (mouse anti-human LAMP-1, U Iowa Hybridoma
Bank). Following PBS wash fixed cells were incubated with HRP-conjugated anti-mouse IgG secondary antibody (ImmPRESS, Vector Laboratories, Burlingame, CA). Following PBS wash detection was performed via TSA upon addition of FITC-conjugated tyramide in Plus Amp Buffer (30 min, RT) followed by PBS wash. Nuclei were next counterstained using bis-benzimide (0.2 µg/ml, Sigma, St. Louis, MO) for 10 min, followed by PBS wash. After coverslipping, co-localization of cathepsin D and LAMP-1 was visualized using a Zeiss Observer.Z1 Laser Scanning Microscope (Thornwood, NY, USA) equipped with a Zeiss™ 40X 1.3 Oil DIC M27 Plan-Apochromat objective and imaged using Zen™ 2008 LSM 710, V5.0 SP1.1 software. Transmitted light images were also taken using DIC optics. Fluorescence filters were used to observe bis benzimide (excitation 405 nm, emission 409–514 nm), FITC (excitation 488 nm, emission 494–572 nm), and Cy3 (excitation 543 nm, emission 585–734 nm).

**Measurement of Lysotracker Red Staining Intensity**

Cells were plated at 1 million per well in a 6-well dish and were treated with DMSO vehicle or rotenone (10 µM) for either 6 or 24h, or bafilomycin A1 (100 nM) for 4h prior to analysis by flow cytometry. Cells were then incubated with LysoTracker® Red (100 nM final concentration, Life Technologies, Eugene, OR) in Locke’s buffer (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO₃, 1.3mM CaCl₂, 1mM MgCl₂, 10mM HEPES, 1.009g/L glucose, pH 7.4) for 1h. Cells were then harvested, pelleted and re-suspended in 1X PBS buffer before being passed through a 70 µm nylon cell strainer (BD Falcon, Durham, NC) into collection tubes to provide a single cell suspension. Cells were kept on ice and protected from light during immediate transport to the flow cytometry facility (Joint UAB Flow Cytometry Facility; Enid Keyser, Director) for analysis. 10,000 events were detected in each experimental condition using the BD
LSR II flow cytometer (Becton Dickinson, San Jose, CA). Further analysis was completed using FlowJo software (Ashland, OR, licensed by UAB). LTR fluorescence was also visualized via microscopy. Subsets of cells were plated in 8 well glass chamber slides (Lab-Tek, Rochester, NY) at a density of 60,000 per well for treatment with vehicle or rotenone (24h), or bafilomycin A1 (4h). Following 1h incubation with LTR was assessed via microscopy using a Zeiss Axioskop® fluorescent microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY) at 40X magnification using bis-benzimide (0.2 µg/ml) for nuclear counter-stain. Transmitted light images were also taken using phase contrast optics. Representative images were collected using AxioVision 4.8 software (Carl Zeiss, Thornwood, NY).

**Statistics**

Significant effects of treatment were analyzed either by Student’s t-test (when two groups were being compared); one-factor ANOVA (when effects of treatment or time were assessed across multiple groups; or by two-factor ANOVA (when the effects of time vs. rotenone treatment were assessed). Post hoc analysis was conducted using Bonferroni’s test. A level of p < 0.05 was considered significant.
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Fig. 1: Rotenone induced SH-SY5Y cell death and caspase 3-like activity are concentration- and time-dependent. Rotenone induced a concentration-dependent decrease in cell viability (A) and increase in caspase 3-like activity (B), observed 24h after treatment. 10µM rotenone induced a time-dependent decrease in SH-SY5Y cell viability (C) that was accompanied by an increase in caspase 3-like activity (D) at 48 and 72h of treatment. Complete inhibition of rotenone-induced
caspase 3-like activity (not shown) with the broad caspase inhibitor Boc-FMK (Boc-Asp-FMK) does not attenuate the decrease in cell viability after 48h treatment with 10 µM rotenone (E). Rotenone (10µM) induced a significant decrease in cellular ATP levels (µM/well) at 6h and 12h after treatment compared to CTL (F). Results represent mean ± standard deviation, and experiments were repeated independently at least three times with similar results.* p<0.05 vs. 0µM rotenone CTL (A, B, E, F); * p<0.05 vs. 0h rotenone (C, D).
Fig. 2: Rotenone causes accumulation of autophagic vacuoles by blocking their effective degradation. (A) Representative western blot of LC3-II (14 kDa) and actin (42 kDa) loading control for SH-SY5Y lysates collected 6h and 24h following treatment with 10 µM rotenone (ROT) in the presence or absence of 100 nM bafilomycin A1 (BafA1). Rotenone caused a significant increase in LC3-II immunoreactivity at both 6h and 24h following treatment (A-C). To measure autophagic flux, 100nM BafA1 was added for the last 2h of rotenone treatment prior
to preparing lysates. Quantification of LC3-II/actin ratios for each treatment is expressed graphically as fold CTL for 6h rotenone (B) and 24h rotenone (C). Treatment with 10µM rotenone induced a significant increase in AV accumulation, an effect that was not significantly greater upon treatment with 100 nM BafA1. Representative western blot indicates that rotenone increase levels of the autophagy substrate p62 at both 6h and 24h after treatment (D). Side bar in (D) indicates higher p62-immunoreactive species following 6h and 24h treatment with rotenone that suggests its enhanced ubiquitination. Blots were stripped and re-probed for actin. Immunoreactivity for p62 (normalized to Actin) is quantified graphically in (E) and is expressed as fold VEH CTL. Results = mean ± standard deviation from 3-5 independent experiments. * p <0.05 vs. VEH CTL.
Fig. 3: Rotenone increases levels of α-synuclein. (A) Representative western blot analysis of whole cell lysates indicates an increase in high molecular weight species of α-synuclein (>50 kDa) following 48h treatment with 10 µM rotenone. (B) Quantification of high-molecular weight species of α-synuclein indicates a significant increase vs. vehicle control. Results = mean ± standard deviation obtained from 3 independent experiments. * p <0.05 vs. VEH CTL.
FIGURE 4

Fig. 4: Rotenone causes an increase in acidic vesicle pH. (A) Representative histogram of alterations in acidic vesicle pH in SH-SY5Y cells after treatment with DMSO vehicle (VEH, 24h, orange line), rotenone (ROT, 10μM, 24h, blue line) or bafilomycin A1 (BafA, 100nM, 4h, pink line) as determined by flow cytometry. Effects of rotenone treatment are expressed as a leftward shift in fluorescence in the cell population when compared to vehicle control, suggesting a loss of lysosomal/acidic vesicle pH. (B) Quantification of LTR mean fluorescence intensity (MFI) indicates a >30% decrease following 6h and 24h treatment with rotenone. (C) Phase contrast and fluorescence microscopy were used to image the rotenone-induced
attenuation of LTR fluorescence at 24h after treatment. Results = mean ± standard deviation from 5 independent experiments * p < 0.05 vs. VEH CTL.

**FIGURE 5**

![Confocal microscopy images](image)

*Fig. 5: Rotenone does not induce lysosomal membrane permeabilization (LMP).* Confocal microscopy images obtained via double label immunocytochemistry for the soluble lysosomal enzyme cathepsin D (red, Cy3) and the lysosomal membrane protein LAMP-1 (green, FITC) following treatment for 24h with DMSO vehicle (top row), 10 µM rotenone (ROT, middle row) or 50 µM chloroquine (CQ, bottom row). Punctate immunoreactivity for cathepsin D that co-localized to regions of the cell exhibiting intense LAMP-1 immunoreactivity was apparent in
vehicle and rotenone-treated cells. Diffuse staining for cathepsin D was observed in chloroquine-treated cells that did not localize intracellularly to that of LAMP-1, suggesting the onset of LMP. Images are representative of LMP assessment from 3 independent experiments.

**FIGURE 6**

**Fig. 6: Rotenone increases levels of LAMP-1.** Representative western blot (A) for LAMP-1 (~110 kDa) along with actin (42 kDa) loading control for lysates obtained from SH-SY5Y cells treated with vehicle control (VEH CTL) or 10 µM rotenone (ROT) for 6h or 24h. Quantification of LAMP-1 signal averaged from 4 independent experiments indicated that levels of LAMP-1 were significantly greater at 6h following rotenone. * p<0.05 vs. vehicle CTL.
Fig. 7 Rotenone exposure does not alter TFEB levels. (A) Representative western blot analysis for TFEB (53kDa) and GAPDH (37kDa) loading control following 6h and 24h treatment with vehicle control (CTL) or 10 µM rotenone (ROT). (B) Quantification of TFEB/GAPDH ratios following 6h and 24h treatment of rotenone are expressed graphically as fold CTL. Results = mean ± standard deviation obtained from 6 independent experiments.
ATP6V0C KNOCKDOWN IN NEUROBLASTOMA CELLS ALTERS AUTOPHAGY-
LYSOSOME PATHWAY FUNCTION AND METABOLISM OF PROTEINS THAT
ACCUMULATE IN NEURODEGENERATIVE DISEASE

by

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ABSTRACT

ATP6V0C is the bafilomycin A1-binding subunit of vacuolar ATPase, an enzyme complex that critically regulates vesicular acidification. We and others have shown previously that bafilomycin A1 regulates cell viability, autophagic flux and metabolism of proteins that accumulate in neurodegenerative disease. To determine the importance of ATP6V0C for autophagy-lysosome pathway function, SH-SY5Y human neuroblastoma cells differentiated to a neuronal phenotype were nucleofected with non-target or ATP6V0C siRNA and following recovery were treated with either vehicle or bafilomycin A1 (0.3-100 nM) for 48h. ATP6V0C knockdown was validated by quantitative RT-PCR and by a significant decrease in Lysostracker® Red staining. ATP6V0C knockdown significantly increased basal levels of microtubule-associated protein light chain 3-II (LC3-II), α-synuclein high molecular weight species and APP C-terminal fragments, and inhibited autophagic flux. Enhanced LC3 and LAMP-1 co-localization following knockdown suggests that autophagic flux was inhibited in part due to lysosomal degradation and not by a block in vesicular fusion. Knockdown of ATP6V0C also sensitized cells to the accumulation of autophagy substrates and a reduction in neurite length following treatment with 1 nM bafilomycin A1, a concentration that did not produce such alterations in non-target control cells. Reduced neurite length and the percentage of propidium iodide-positive dead cells were also significantly greater following treatment with 3 nM bafilomycin A1. Together these results indicate a role for ATP6V0C in maintaining constitutive and stress-induced ALP function, in particular the metabolism of substrates that accumulate in age-related neurodegenerative disease and may contribute to disease pathogenesis.
INTRODUCTION

Vacuolar-ATPase (V-ATPase) is a membrane-associated, multi-subunit protein complex that functions as an ATP-driven proton-pump. V-ATPase is organized into two coordinately operating multi-subunit domains: the peripheral V₁ domain that performs ATP hydrolysis and the integral V₀ domain that allows for proton translocation across the membrane. The V₁ and V₀ domains are connected to each other by a central “stalk” of shared subunits. The rotary action of the stalk subunits has been proposed to drive proton translocation across the membrane upon V₁ hydrolysis of ATP.

V-ATPase is localized to many different membranes of eukaryotic cells including lysosomes, endosomes, Golgi-derived vesicles, secretory vesicles and in some cell types the plasma membrane. V-ATPase has well documented functions, including maintenance of both acidic vesicle and cytosolic pH and vesicle fusion with vacuoles. V-ATPase-dependent maintenance of acidic pH in lysosomes and endosomes is important for optimal function of their proteolytic enzymes, whereas V-ATPase-dependent vesicle fusion serves a variety of functions including neurotransmitter release from synaptic vesicles, transport of Golgi-derived lysosomal enzymes and membrane proteins, and effective fusion of autophagosomes with lysosomes and endosomes.

Pharmacologic inhibition of V-ATPase was first reported in 1988 by the use of antibiotic drugs coined “bafilomycins” derived from Streptomyces soil bacteria. Bafilomycin A1 and structurally related compounds have in common a 16-18 membered macrolactone ring linked to a unique side chain and together represent the plecomacrolide subclass of macrolide antibiotics. Bafilomycin A1 has been shown to inhibit V-ATPase with high affinity, at concentrations ≥ 10nM. Bafilomycin A1 and similarly structured compounds are widely used as pharmacologic...
tools to inhibit lysosome acidification and inhibit autophagy-lysosome pathway (ALP) function by preventing autophagosome-lysosome fusion, thus promoting the robust accumulation of autophagosomes. It is believed that V-ATPase-dependent vesicle fusion also requires the maintenance of acidic pH, though recent studies have indicated that fusion may occur in a pH-independent manner.

It is widely believed that inhibition of ALP function contributes to the aberrant accumulation of protein species in age-related neurodegenerative disease that not only define disease-specific neuropathology but also may contribute to disease pathogenesis, including α-synuclein in Parkinson’s disease and metabolites of amyloid precursor protein (APP) in Alzheimer’s disease. Several ALP-associated molecules have been identified that are currently being investigated for their utility as therapeutic targets in age-related neurodegenerative disease. Experimental inhibition of V-ATPase by bafilomycin A1 prevents the effective degradation of α-synuclein that in turn promotes accumulation of α-synuclein soluble oligomeric and insoluble aggregate species with neurotoxic potential. Bafilomycin A1-mediated inhibition of V-ATPase also effectively inhibits the rapid degradation of full-length APP and its metabolites, C-terminal fragments (CTFs) that are formed initially upon cleavage of full-length APP by β-secretase. Subsequent cleavage by γ-secretase can promote the generation of toxic Aβ species, whereas subsequent cleavage by α-secretase or γ-secretase can generate the putatively toxic APP intracellular domain (AICD). As such, the ALP and putatively V-ATPase represent attractive targets for promoting the metabolism of proteins that contribute to the pathogenesis of age-related neurodegenerative disease.
Through analysis of bafilomycin A1-resistant strains of the fungus *Neurospora crassa* it was discovered that bafilomycin A1 inhibition of V-ATPase activity is mediated by binding with high affinity to the c subunit in the V\(_0\) domain, or ATP6V0C\(^3\). Knockdown of ATP6V0C has been shown recently to inhibit vesicular acidification and sensitize cells to stress-induced cell death\(^6,7,51\), while ATP6V0C-deficient mice are embryonic lethal\(^42\). However, whether ATP6V0C itself is responsible for regulating ALP function, as well as the metabolism of substrates that accumulate in age-related neurodegenerative diseases has not been previously investigated. In the present study we found that knockdown of ATP6V0C in neuronal cells adversely affected ALP function concomitant with accumulation of ALP-associated substrates including α-synuclein and APP-CTFs, and exacerbated stress-induced cell death. Our findings suggest an important role for ATP6V0C in maintenance of ALP function that may portend relevance to age-related neurodegenerative disease.

**MATERIALS AND METHODS**

**Cell Culture**

Naïve SH-SY5Y human neuroblastoma cells (ATCC, CRL-2266) were maintained in T-75 flasks (Corning, 430641) at 37°C and 5% CO\(_2\) in Minimum Essential Media (Cellgro, 10-010-CV) and F12-K media (ATCC, 30-2004) supplemented with 0.5% sodium pyruvate (Cellgro, 25-000-CI), 0.5% non-essential amino acids (Cellgro, 25-025-CI), 1% penicillin/streptomycin (Invitrogen, 15140-122), and 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific, SH30109.03). Naïve cells received full media change every two days. Prior to their use in experiments, naïve SH-SY5Y cells were differentiated for seven days to a post-mitotic state in
10% FBS media supplemented with 10 μM retinoic acid (RA; Sigma, R2625). Complete RA-supplemented media was replenished every two days.

**Nucleofection with siRNA**

Small-interfering RNA (siRNA) specific for human ATP6V0C was obtained from Thermo Scientific. The Amaxa Nucleofector™ system (Lonza, VVCA-1003), a modified electroporation system, was utilized to transiently knock down ATP6V0C. First, RA-differentiated SH-SY5Y cells were re-suspended in the Lonza™ Nucleofector reagent. Next, 400nM of re-suspended ATP6V0C siRNA (Thermo Scientific, M-017620-02-0010), or non-target siRNA (Thermo Scientific, D-001206-13-05), (in siRNA buffer with 20 mM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM MgCl₂) was added to the cell suspension. After electroporation, 500 μL of FBS-containing media was immediately added to the cell suspension. Following a 10 min recovery period, nucleofected cells were transferred to T-75 flasks and incubated overnight in 10% FBS differentiation media for 24h. The next day nucleofected cells were plated for experiments (24h after nucleofection and 24h prior to drug treatment) in either eight well glass chamber slides (LabTek, 154941), six well plates (Corning 3516) or 60 mm dishes (Corning, 430166) at a density of 500/mm² in 0% FBS differentiation media containing 2% B-27 supplement (Invitrogen, 17504044). All experimental endpoints ended at 96h after nucleofection.

**Treatment with Bafilomycin A1**

Following nucleofection, recovery and plating for experiments (i.e. 48h after nucleofection), media was exchanged for fresh 0% FBS differentiation media containing 2% B-
27 supplement with either DMSO vehicle (0 nM control) or bafilomycin A1 (0.3-100 nM; AG Scientific, B-1183). Bafilomycin A1 was prepared as a 10 mM stock solution in DMSO (Sigma, D8418-1L) and stored at -20°C. Unless otherwise noted, all bafilomycin A1 treatments were for 48h, with experiments ending 96h after initial nucleofection.

**Quantitative Real Time PCR**

Cells nucleofected with ATP6V0C siRNA or non-target siRNA were processed for total RNA extraction with the use of TRIzol reagent (Invitrogen, 15596-026). cDNA was generated with oligo (dT) from four µg of RNA using the SuperScript III First Strand Synthesis System (Invitrogen #18080-051). Primers used for detection of ATP6V0C mRNA were ATP6VOC 5’-ATGTCCGAGTCCAAGAGCGGC-3’ and ATP6VOC 5’-CTACTTTGTGGAGAGGATGAG-3’. Primers for actin were 5’-GCTCGTCGTCGACAACGGCTC-3’ and 5’-CAAACATGATCTGGTCATCTTCTC-3’. Template for cDNA (2 µL) was added along with 1X Fast SYBR Green Master Mix (ABI #4385610) and water to a final volume of 20µL/well. Assays were performed in triplicate on a Bio-Rad CFX96 instrument using the following conditions: (95°C for three minutes followed by 95°C for 15 seconds and 60°C for one minute for 45 cycles. The comparative cycle threshold (Ct) method (ddCT; 14) was used to determine expression of ATP6V0C in each sample relative to endogenous actin control, which was then used to determine knockdown in ATP6V0C siRNA samples as a percentage of non-target siRNA control.
**Measurement of Acidic Vesicle pH**

After treatment for 48h with DMSO vehicle, cells plated in 60 mm dishes were incubated with LysoTracker Red® (LTR; Life Technologies™, L7528; 100 nM final concentration) for 30 min in Locke’s buffer (15mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1.3mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 1.009 g/L glucose, pH 7.4). Cells were then harvested, pelleted, and re-suspended in 1 X PBS then passed through a 70 μm nylon cell strainer (BD Falcon, 08-771-2) and into collection tubes to provide a single cell suspension. Cells were kept on ice and protected from light during immediate transport to the Joint UAB Flow Cytometry Facility (Enid Keyser, Director) for analysis. A total of $1 \times 10^4$ events were detected in each experimental condition using the BD LSR II flow cytometer (Becton Dickinson). Further analysis was performed using FlowJo software (licensed by UAB) to assess mean fluorescence intensity. LTR fluorescence was also visualized via microscopy in cells plated in eight well glass chamber slides and treated for either 48h with DMSO vehicle or with 100 nM bafilomycin A1 for the last 3h of the 48h time course. Following treatment with LTR, fluorescent images were captured using a Zeiss Axioskop fluorescent microscope (Carl Zeiss Micro-imaging, LLC) at 40X magnification. Transmitted light images were also taken using phase contrast optics.

**Western Blot Analysis**

After treatment for 48h, nucleofected cells in 60 mm dishes were incubated for seven minutes at 37°C with Accutase (Innovative Cell Tech., AT104) to detach cells from their substrate. Whole cell lysates were collected and protein concentrations were determined using BCA protein assay (Fisher Scientific, PI-23227) as previously described. Equal amounts of each protein sample were then electrophoresed using 12% Tris/Glycine SDS-polyacrylamide
gels, or for APP CTFs using 16.5% Tris-Tricine pre-cast gels (Bio-Rad Laboratories, 456-3064) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, 162-0177).

Following a 30 minute incubation at room temperature with 5% blocking milk (Bio-Rad, 170-6404) in 1X tris-buffered saline with tween (TBST), membranes were incubated overnight with primary antibodies for immunodetection of the following proteins: LAMP1 (University of Iowa Hybridoma Bank, H4A3); LC3 (rabbit anti-LC3, Abcam, ab51520); α-syn (Santa Cruz Biotechnology, Inc., SC7011); and APP CTF (Covance Inc, SIG-39152). Membranes were washed with 1X TBST containing 0.1% Tween 20 and then incubated with secondary IgG-HRP conjugated antibody for 1h at room temperature. After washing blots, SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, PI-34096) was used to detect α-syn and Enhanced chemiluminescence (ECL; Thermo Scientific, PI-32106) was used to detect LAMP-1, LC3 and APP. Blots were then stripped with Restore™ Western Blot stripping buffer (Thermo Scientific, 21059) and probed for actin (Sigma, A1978) to normalize for gel loading. Films with detected bands of interest were scanned using Adobe® Photoshop® and band intensities were calculated using UN-SCAN-IT gel digitizing software (Silk Scientific, Inc.).

**Assessment of Autophagic Flux**

Following nucleofection, plating for experiments and 44-46h following media change, subsets of cells in six well plates were treated with 100 µM chloroquine (Sigma, C6628) plus 200 µM leupeptin (Sigma, L9783) to inhibit lysosome function. At 48h following initial media change (equaling 2-4h following treatment with lysosome inhibitors) lysates were processed as above for western blot analysis of LC3-II and actin loading control.
Assessment of Autophagosome-Lysosome Fusion

Following nucleofection with ATP6V0C siRNA or non-target control siRNA, cells plated in eight well glass chamber slides were treated for 48h with DMSO and subsequently fixed for 15 min at 4°C using Bouin’s fixative (75% saturated picric acid, 23.8% of a 37% w/v formaldehyde solution, 4.7% glacial acetic acid) in the presence of 0.5% saponin (Sigma, 47036). Following incubation for 30 min with 1X PBS blocking buffer containing 1% bovine serum albumin, 0.2% non-fat dry milk and 0.3% Triton X-100, cells were incubated overnight at 4°C in blocking buffer without Triton X-100 and containing rabbit anti-LC3 antibody (Sigma, L7543) to detect autophagosome punctae. Following PBS wash, fixed cells were incubated for 1h with SuperPicTure™ (Invitrogen, 879263) anti-rabbit IgG secondary antibody. Following PBS wash, fixed cells were subjected to tyramide signal amplification (incubation for 30 min with Cy3 plus tyramide, Perkin Elmer, FP1170) to detect LC3. Next, fixed cells were incubated for 10 min with 3% hydrogen peroxide to neutralize residual peroxidase from the first secondary antibody. After PBS wash and 30 min incubation with blocking buffer, fixed cells were incubated overnight at 4°C in blocking buffer containing the second primary antibody, mouse anti-human LAMP-1 (University of Iowa Hybridoma Bank, H4A3) to detect lysosomes. Following subsequent PBS wash, fixed cells were incubated for 1h with Vector Impress (Vector Labs, MP-7402) anti-mouse IgG. Following PBS wash, tyramide signal amplification was performed (incubation for 30 min with FITC plus tyramide, Perkin Elmer, FP1168) to label LAMP-1. Following PBS wash, fixed cells were last incubated with bis-benzimide (0.2 µg/ml in PBS, Sigma) to label nuclei. Images were captured using a Zeiss Observer.Z1 laser scanning microscope equipped with a Zeiss 40X Plan-Achromat objective and imaged using Zen 2008 LSM 710, V5.0 SP1.1 software.
**Assessment of Cell Death Morphology and Neurite Length**

After treatment for 48h with 0-10 nM bafilomycin A1, nucleofected cells plated on eight well glass chamber slides were fixed with Bouin’s fixative for 15 min, 4°C. Cell death morphology was visualized via transmitted light and DIC optics on a Zeiss Observer.Z1 laser scanning microscope equipped with a Zeiss 40X 1.3 DIC M27 Plan-Apochromat objective and imaged using Zen 2008 LSM 710, V5.0 SP1.1 software. Neurite length was assessed by first capturing images using Olympus BX51 microscope (Olympus of the Americas) and a 100X UPlan FLN objective. Neurites were measured using the tracing tool within Neurolucida software (MBF Bioscience, version 5.65). Cells identified for measurements had neuritic processes that did not form a synapse with neighboring cells. Data files collected using Neurolucida were quantified using the Nueurolucida Explorer program to determine average neurite length per cell.

**Quantification of Percent Cell Death**

Cell death was quantified using propidium iodide (PI; Life Technologies, V13242) staining and flow cytometry. Following treatment for 48h cells with 0-100 nM bafilomycin A1, nucleofected cells were incubated with PI for 15 min at a final concentration of 1μg/mL. Cells were then prepared for flow cytometry as above for assessment of acidic vesicle pH and quantified as the percentage of cells that were PI-positive relative to vehicle control using FlowJo software.
**Statistical Analysis and Figure Preparation**

Quantitative real time PCR data were expressed as mean ± SEM percent knockdown for ATP6V0C. LTR staining was presented as mean ± SEM fluorescence intensity. Western blot band intensities for LAMP-1, LC3-II, α-syn and APP-CTFS were normalized to actin loading control, and were expressed (mean ± SEM) either as such to assess bafilomycin A1 concentration responsiveness, or further normalized for each ATP6V0C siRNA condition as mean ± SEM fold change relative to its companion non-target control to determine effects of genetic manipulation. Neurite length was presented as mean ± SEM average length (µm) per cell. Cell death was quantified as mean ± SEM percentage of PI-positive cells. To determine effects of genetic manipulation, between groups comparisons of Non-target vs. ATP6V0C siRNA were evaluated for significance using either one-sample t-test (LTR data; quantitative real-time PCR data; western blot data normalized as fold change relative to the companion non-target control) or two-sample t-test (assessment of neurite length and percent cell death). In addition, to assess concentration responsiveness, within group comparisons of absolute (raw) data were evaluated for significance using one-way ANOVA. Significant ANOVAs were followed by post hoc analysis using Bonferroni’s Multiple Comparison Test. For all tests, statistical significance was set *a priori* at p < 0.05. Graph Pad Prism® was used to perform statistical analysis and generate graphs, and Adobe® Photoshop® was used to assemble figures. A minimum of three independent replicates were performed for each experimental endpoint.
RESULTS

Validation of ATP6V0C knockdown

We first set out to confirm siRNA-mediated knockdown of ATP6V0C. Quantitative real-time PCR analysis was performed on cDNA that was generated from RNA in samples isolated from differentiated SH-SY5Y human neuroblastoma cells harvested 96h following nucleofection with non-target or ATP6V0C siRNA. Using the comparative cycle threshold (Ct) method (ddCT; \(^{14}\)) with actin mRNA expression serving as an internal standard, we determined that ATP6V0C mRNA was significantly reduced by \(95.01 \pm 2.33\%\) (\(p<0.05\) vs. Non-target siRNA using one sample t-test) in ATP6V0C siRNA samples compared to non-target control (Table 1).

Table 1

Expression of ATP6V0C by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amplicon</th>
<th>Ct(^1)</th>
<th>dCt(^2)</th>
<th>ddCt(^3)</th>
<th>% ATP6V0C expression(^4)</th>
<th>% knockdown(^5)</th>
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<tbody>
<tr>
<td>ATP6V0C siRNA</td>
<td>ATP6V0C</td>
<td>27.58</td>
<td>4.99</td>
<td>4.32</td>
<td>4.99%</td>
<td>95.01% +/- 2.33%</td>
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<tr>
<td>ATP6V0C siRNA</td>
<td>Actin</td>
<td>22.59</td>
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<tr>
<td>Non-target siRNA</td>
<td>ATP6V0C</td>
<td>24.29</td>
<td>0.67</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Non-target siRNA</td>
<td>Actin</td>
<td>23.62</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(^1\) Ct is number of cycles to reach threshold, or threshold cycle
\(^2\) dCt = average Ct(ATP6V0C) – average Ct(Actin)
\(^3\) ddCt = dCt(ATP6V0C siRNA) – dCt(Non-target siRNA)
\(^4\) % ATP6V0C expression = \(2^{-\text{ddCt}}\)
\(^5\) % Knockdown = 100% - 4.99%

*\(p<0.05\) via one-sample t-test compared to Non-target siRNA control; \(n=3\) replicates

To determine if ATP6V0C knockdown regulates vesicular acidification as previously reported \(^{51}\) we performed flow cytometric analysis of LTR staining at 96h following
nucleofection (Fig. 1). Representative images indicated a noticeable reduction in LTR fluorescence following knockdown with ATP6V0C siRNA following vehicle treatment (Fig. 1A, B) compared to non-target control (Fig. 1D, E). As a comparative control, treatment for 3h with 100 nM bafilomycin A1 appeared to completely ameliorate LTR fluorescence for both non-target control cells and following knockdown with ATP6V0C (Fig. 1C, 1F). Quantification of LTR mean fluorescence intensity in vehicle-treated cells (Fig. 1G) indicated a significant reduction following ATP6V0C knockdown (0.54 ± 0.04) expressed as fold-change relative to non-target control (Fig. 1B), confirming in our knockdown model the importance of ATP6V0C for the maintenance of acidic vesicle pH.

**ATP6V0C knockdown increases levels of lysosome marker LAMP-1 levels by a low concentration of bafilomycin A1**

Levels of LAMP-1 (lysosome-associated membrane protein-1) have been shown previously to increase following lysosomal stress. To further determine the effects of ATP6V0C knockdown on acidic vesicles we performed western blot analysis to assess LAMP-1 levels at 48h following treatment (Fig. 2A, C, E). Levels of LAMP-1 were assessed following treatment with vehicle control or following treatment with bafilomycin A1 at concentrations shown previously to have either little effect on inhibiting V-ATPase or vesicular pH (1 nM) or cause profound inhibition of V-ATPase and vesicular acidification at ≥10 nM. In non-target siRNA control cells, significant increases in LAMP-1/actin ratios were observed following treatment with 10 nM (2.89 ± 0.41) and 100 nM (2.92 ± 0.39) bafilomycin A1 compared to treatment with 0 nM (0.90 ± 0.23) or 1 nM (1.22 ± 0.31) concentrations (Fig. 2C, open columns). In ATP6V0C siRNA cells (Fig. 2C, filled columns), the concentration responsiveness of
bafilomycin A1 shifted such that significantly higher LAMP-1/actin ratios were observed at all three concentrations tested (1 nM: 2.03 ± 0.28; 10 nM: 2.52 ± 0.33; 100 nM: 2.18 ± 0.32) compared to that observed in 0 nM vehicle control (0.76 ± 0.18). When effects of ATP6V0C siRNA knockdown were compared directly to that of non-target siRNA control (Fig. 2E), a significant 2.06 ± 0.30 fold increase in relative LAMP-1/actin was observed at the 1 nM concentration, whereas fold changes by 0 nM vehicle control (0.89 ± 0.16), 10 nM (1.03 ± 0.24) or 100 nM (0.79 ± 0.11) concentrations of bafilomycin A1 were not significantly different from non-target control. Together these findings indicate that ATP6V0C knockdown shifted the concentration responsiveness of cells to bafilomycin A1 by increasing LAMP-1 levels at a lower concentration not ordinarily associated with lysosome inhibition.

**ATP6V0C knockdown increases basal and stress-induced accumulation of autophagosomes.**

To determine if ATP6V0C deficiency affected the basal vs. bafilomycin A1-induced accumulation of autophagosomes we performed western blot analysis for LC3-II (Fig. 2B, D, F). Assessing bafilomycin A1 concentration responsiveness in non-target siRNA control cells (Fig. 2D, open columns), treatment with 10 and 100 nM bafilomycin A1 produced significantly greater LC3-II/actin ratios (10 nM: 1.99 ± 0.45; 100 nM: 2.16 ± 0.36) compared to 0 nM vehicle control (0.40 ± 0.10), while LC3-II/actin ratios following treatment with 1 nM bafilomycin A1 (0.79 ± 0.10) were significantly different only in comparison to the 100 nM concentration.

While a significant one-way ANOVA was obtained in assessing the bafilomycin A1 concentration responsiveness resulting from ATP6V0C knockdown (Fig. 2D, filled columns), post hoc analysis revealed no significant differences in LC3-II/actin ratios in knockdown cells with respect to any concentration of bafilomycin A1. When effects of ATP6V0C siRNA
knockdown were compared directly to that of non-target siRNA control (Fig. 2E), treatment with both 0 nM vehicle control (1.55 ± 0.10) and 1 nM bafilomycin A1 (2.36 ± 0.39) exhibited significantly higher fold changes in relative LC3-II/actin in ATP6V0C knockdown cells compared to non-target siRNA control. Fold change differences in relative LC3-II/actin following ATP6V0C knockdown and treatment with 10 nM bafilomycin A1 (1.08 ± 0.09) or 100 nM bafilomycin A1 (1.01 ± 0.09) were not significantly different relative to non-target siRNA control. These findings indicate that ATP6V0C knockdown not only increased the basal accumulation of autophagosomes but also enhanced their accumulation at a “low” concentration of bafilomycin A1. The lack of significant concentration responsiveness following ATP6V0C knockdown may reflect the observed increase in autophagosomes following 0 nM vehicle and 1 nM bafilomycin A1 treatment.

**ATP6V0C knockdown inhibits autophagic flux**

To determine the manner by which knockdown of ATP6V0C caused the accumulation of autophagosomes we assessed autophagic flux by western blot analysis of LC3-II, following inhibition of lysosome function for the last 2-4h of a 48h treatment period by treatment with a high concentration of the lysosomotropic agent and weak base chloroquine, and the protease inhibitor leupeptin (Fig. 3A-B) as previously reported. Treatment of non-target siRNA control cells with lysosome inhibitors caused a robust and significant increase in LC3-II/actin (1.39 ± 0.26) compared to vehicle treatment alone (0.69 ± 0.06). When the effects of ATP6V0C siRNA knockdown were compared relative to non-target siRNA control, a significant fold-change increase was observed following vehicle treatment (1.51 ± 0.19), while the fold-change difference in relative LC3-II/actin following treatment with lysosome inhibitors for
ATP6V0C siRNA knockdown (1.14 ± 0.13) was not significantly different from non-target control. To further investigate the manner by which autophagic flux was inhibited we performed double-label immunocytochemistry for LC3 and LAMP-1 in vehicle-treated non-target siRNA control cells (Fig. 3C-E) and ATP6V0C siRNA knockdown cells (Fig. 3F-H). Representative images from vehicle-treated non-target siRNA control cells indicate a relative lack of co-localization with LC3 and LAMP-1 punctae (Fig. 3E). In comparison, there were several instances of LC3 and LAMP-1 punctae co-localizing in vehicle treated ATP6V0C knockdown cells (Fig. 3H). Together these results suggest that ATP6C0C knockdown inhibits autophagic flux in a manner caused in part by the accumulation of dysfunctional LC3 and LAMP-1-positive autolysosomes.

**ATP6V0C knockdown induces basal and stressed accumulation of high molecular weight α-synuclein species**

It is well-established that lysosome function is important for the effective metabolism of substrates including α-synuclein that accumulate in age-related neurodegenerative diseases. We have shown previously that treatment of differentiated SH-SY5Y cells with agents that disrupt ALP function causes the accumulation of high molecular weight (MW) α-synuclein species that are associated with PD pathology. Specifically, inhibition of V-ATPase with bafilomycin A1 has been shown using western blot analysis to increase levels of α-synuclein high MW species. Thus western blot analysis was used to determine if knockdown of ATP6V0C altered levels of endogenous high MW α-synuclein species (defined as greater than 50 kDa) under basal (vehicle) conditions or following treatment with 1-100 nM bafilomycin A1 (Fig. 4). Representative western blot analysis suggests that treatment with bafilomycin A1
induced the accumulation of α-synuclein high MW species (Fig. 4A). However, one-way ANOVA of pooled data (Fig. 4B) was not significant, thus suggesting a lack of concentration dependence between groups of non-target siRNA control cells treated with 0 nM (2.74 ± 0.81), 1 nM (3.24 ± 0.81), 10 nM (4.98 ± 1.53) or 100 nM (5.46 ± 1.55) bafilomycin A1, or between groups of ATP6V0C siRNA knockdown cells treated with 0 nM (3.68 ± 0.87), 1 nM (4.38 ± 1.17), 10 nM (4.94 ± 1.40) or 100 nM (4.65 ± 1.02) bafilomycin A1. This lack of significant concentration-dependence, at least for non-target siRNA control cells may be explained by the wide range in absolute values for α-synuclein/actin ratios observed between different experiments. Regardless, when effects of ATP6V0C siRNA knockdown were compared directly to that of non-target siRNA control (Fig. 4D), treatment with both 0 nM vehicle control (1.90 ± 0.38) and 1 nM bafilomycin A1 (1.75 ± 0.32) exhibited significantly higher fold changes in relative α-synuclein high MW species/actin compared to non-target siRNA control. Fold changes between observed following treatment with 10 nM (1.31 ± 0.24) or 100 nM (1.12 ± 0.16) bafilomycin A1 were not significantly different. Levels of α-synuclein monomer (17 kDa) were also assessed in non-target siRNA control and ATP6V0C siRNA knockdown cells (Fig. 4A). Similar to α-synuclein high MW species (Fig. 4B), one-way ANOVA was not significant, suggesting a lack of bafilomycin A1 concentration responsiveness for ratios of α-synuclein monomer/actin (Fig. 4C) between groups of non-target siRNA control cells treated with 0 nM (0.79 ± 0.19), 1 nM (0.95 ± 0.21), 10 nM (0.91 ± 0.21) or 100 nM (0.89 ± 0.21) bafilomycin A1, or between groups of ATP6V0C siRNA knockdown cells treated with 0 nM (1.03 ± 0.23), 1 nM (1.12 ± 0.25), 10 nM (0.78 ± 0.20) or 100 nM (0.65 ± 0.18) bafilomycin A1. Interestingly, when the effects of ATP6V0C siRNA knockdown were compared directly to that of non-target siRNA control (Fig. 4E), a significant fold-change decrease in α-synuclein monomer was observed in ATP6V0C
siRNA knockdown cells treated with 100 nM bafilomycin A1 (0.75 ± 0.10), even though concomitant increases in α-synuclein high MW species were not observed at this concentration. Significant differences in α-synuclein monomer were not observed in ATP6V0C knockdown cells treated with vehicle control (0 nM: 1.38 ± 0.19) or other concentrations of bafilomycin A1 (1 nM: 1.21 ± 0.10; 10 nM: 0.95 ± 0.13). In summary, while treatment with bafilomycin A1 did not cause concentration-dependent increases in α-synuclein, our findings suggest that ATP6V0C deficiency alters both the basal and stress-induced metabolism of α-synuclein.

**ATP6V0C knockdown induces basal and stress-induced accumulation of APP CTFs**

The importance of intact ALP function for the effective degradation of APP and its enzymatic cleavage products, termed C-terminal fragments (CTFs) is also well documented. Treatment with bafilomycin A1 and other inhibitors of lysosome function have been shown previously to promote the accumulation of APP CTFs. To determine if ATP6V0C regulated the basal vs. stressed accumulation of endogenous APP CTFs we performed western blot analysis using an antibody that recognizes both full-length APP and its CTFs (Fig. 5). CTFs were defined in our study as ≤ 15 kDa based on previously published reports and were quantified as such. APP CTFs observed in our study correspond in size to the C99 fragment (14 kDa), formed by sequential β- and γ-secretase cleavage of APP in the “amyloidogenic” pathway; the C83 fragment (12.5 kDa), formed by β- and α-secretase cleavage of APP in the “non-amyloidogenic” pathway; and the APP intracellular domain (AICD), formed by both amyloidogenic and non-amyloidogenic pathways. Bafilomycin A1 concentration responsiveness was first assessed for ratios of APP-CTFs/actin (Fig. 5B) in non-target siRNA control cells (open columns) and ATP6V0C siRNA knockdown cells (filled columns). Results
of one-way ANOVA and post-hoc analysis revealed significant increases in ratios of APP CTFs/actin following treatment with bafilomycin A1 at 10 nM (5.15 ± 0.62) and 100 nM (5.71 ± 1.16) concentrations, compared to that of treatment with either 0 nM (0.47 ± 0.16) or 1 nM (0.76 ± 0.32) concentrations. For ATP6V0C siRNA knockdown cells, significant differences in fold-change APP CTFs/actin were observed at 10 nM (5.33 ± 1.23) and 100 nM (5.31 ± 1.45) concentrations of bafilomycin A1 when compared to those in the 0 nM vehicle control group (0.74 ± 0.22) but not following treatment with 1 nM bafilomycin A1 (2.13± 0.60). When the effects of ATP6V0C siRNA knockdown were compared directly to non-target siRNA control (Fig. 5C), significant increases in ratios of APP CTFs/actin relative to siRNA control were revealed for both 0 nM (1.64 ± 0.12) and 1 nM (3.33 ± 0.71) concentrations of bafilomycin A1, while comparisons between 10 nM (0.99 ± 0.13) and 100 nM (0.92 ± 0.16) concentrations were not significantly different. These results suggest that ATP6V0C plays a functional role in the effective degradation of APP CTFs, some of which exhibit documented neurotoxic potential.

**ATP6V0C deficiency induced markers of neurotoxicity.**

As ATP6V0C deficiency was shown previously to exacerbate stress-induced cell death \(^6,^{7,51}\) we next determined if knockdown of ATP6V0C was similarly toxic to differentiated SH-SY5Y neuroblastoma cells by investigating different markers of cytotoxicity (Fig. 6). Representative cell morphology is pictured for non-target siRNA cells (Fig. 6A-D) or for ATP6V0C siRNA knockdown cells (Fig. 6E-H). DMSO vehicle-treated non-target control cells (Fig. 6A) and ATP6V0C knockdown cells (Fig. 6E) both exhibited normal appearing cell soma and neurites. Neuritic processes imaged in our study appear similar to those observed previously in SH-SY5Y cells \(^{26,33}\). Non-target control cells treated with 1 nM bafilomycin A1 (Fig. 6B)
also appeared morphologically similar to vehicle-treated non-target cells (Fig. 6A), but looked healthier than ATP6V0C knockdown cells treated with 1 nM bafilomycin A1 (Fig. 6F), which exhibited comparatively shorter neurites. Treatment with 3 nM bafilomycin A1 caused a noticeable reduction in neurite length as well as increased pyknosis in both non-target control cells (Fig. 6C) and ATP6V0C knockdown cells (Fig. 6G), although neurite length in ATP6V0C knockdown cells treated with 3 nM bafilomycin A1 appeared shorter than non-target control cells treated at the same concentration. Treatment with 10 nM bafilomycin A1 caused pronounced pyknosis and neurite loss in both non-target control cells (Fig. 6D) and ATP6V0C knockdown cells (Fig. 6H). To quantify our morphological observations we measured neurite length (Fig. 6I). One-way ANOVA was performed followed by post hoc analysis to determine bafilomycin A1 concentration responsiveness for non-target siRNA control-treated cells (Fig. 6I, open columns) or ATP6V0C siRNA knockdown cells (Fig. 6I, filled columns). For non-target siRNA control cells, vehicle control neurite length (87.77 ± 7.80) was significantly greater than that observed at 3 nM (55.22 ± 4.38) or 10 nM (23.58 ± 2.35). Neurite length in non-target control cells following treatment with 10 nM bafilomycin A1 was also significantly less than in cells treated with 1 nM (70.42 ± 6.18) or 3 nM bafilomycin A1. In ATPV0C knockdown cells, neurite length following treatment with DMSO vehicle (67.15 ± 6.54) was significantly greater than that measured following treatment with all other concentrations of bafilomycin A1 (1 nM: 48.38 ± 5.38; 10 nM: 21.61 ± 1.61; 10 nM: 20.29 ± 1.67). In addition, neurite length in 1 nM bafilomycin A1-treated cells was significantly greater than that of 3 and 10 nM concentrations. When neurite lengths in ATP6V0C knockdown cells were compared to those in non-target control cells using two-sample t-test, significant reductions in neurite lengths were observed in
ATP6V0C knockdown cells at 0 nM, 1 nM and 3 nM concentrations of bafilomycin A1 but not at the 10 nM concentration.

Flow cytometric analysis was also used to quantify the effects of ATP6V0C deficiency on cell death by assessing the percentage of PI-positive cells that accumulated 48h after treatment with DMSO vehicle or 0.3, 1, 3, 10 or 100 nM bafilomycin A1 (Fig. 6J). Percent cell death (mean ± SEM) was measured as follows for non-target control cells (open columns):

DMSO vehicle: 16.75 ± 2.24; 0.3 nM: 18.94 ± 2.09; 1 nM: 20.35 ± 2.36; 3 nM: 37.30 ± 4.10; 10 nM: 81.17 ± 0.36; 100 nM: 81.3 ± 1.48. Following a significant one-way ANOVA and subsequent post hoc analysis, significant increases in percent cell death were observed following treatment with concentrations ≥ 3 nM bafilomycin A1 compared to treatment with 0 nM vehicle control or 0.3 and 1 nM concentrations. Percent cell death following treatment with bafilomycin A1 was also significantly greater at 10 and 100 nM compared to treatment with 3 nM. Percent cell death for ATP6V0C knockdown cells (filled columns) were as follows: DMSO vehicle: 17.31 ± 2.07; 0.3 nM: 23.30 ± 1.08; 1 nM: 27.97 ± 4.89; 3 nM: 66.98 ± 5.63; 10 nM: 79.07 ± 2.48; 100 nM: 82.57 ± 2.42. Similar to non-target control, results of one-way ANOVA were found to be significant, with post hoc analysis indicating significant increases in percent cell death following treatment with concentrations ≥ 3 nM bafilomycin A1, compared to treatment with 0 nM vehicle control or 0.3 nM and 1 nM concentrations. However, unlike non-target siRNA-treated cells, percent cell death in 3 nM bafilomycin A1-treated ATP6V0C knockdown cells treated was not different than that observed at 10-100 nM concentrations. Asterisks in Fig. 6J indicate significant differences with respect to concentration of bafilomycin A1. When percent cell death following ATP6V0C knockdown was compared directly to non-target control using two-sample t-test, a significant increase in percent cell death was observed specifically in
ATP6V0C knockdown cells treated with 3 nM bafilomycin A1, as indicated by the hash tag (#) in Fig. 6J. Together these findings corroborate our other findings by indicating an increased sensitivity of cells to bafilomycin A1-induced cytotoxicity following knockdown of ATP6V0C.

**DISCUSSION**

V-ATPase regulates the proper function of acidic vesicles including endosomes and lysosomes, in part through its maintenance of vesicular pH. This is important not only for the effective transport and delivery of lysosome-associated structural and functional molecules to their pre-destined location, but also for protein degradation pathways that rely on the effective fusion between endosomes, autophagosomes and/or lysosomes. While several studies have indicated the ability of V-ATPase inhibitors including bafilomycin A1 to regulate autophagy-lysosome pathway function and substrate degradation, it is unknown whether the molecular target that bafilomycin A1 binds, the ATP6V0C subunit of V-ATPase, directly mediates these events. Results of the present study show how knockdown of ATP6V0C increases vesicular pH and inhibits basal autophagic flux as well as basal and stress-induced metabolism of autophagy markers/substrates, suggesting that ATP6V0C contributes to the function of the autophagy-lysosome pathway under constitutive conditions and under conditions of stress. In addition, results of our cytotoxicity assays indicate the contribution of ATP6V0C in the maintenance of cell survival under basal and stressed conditions.

Basal vesicular acidification was significantly attenuated following knockdown of ATP6V0C in SH-SY5Y cells differentiated to a neuronal phenotype, which is similar to previous findings in MCF-7 breast cancer tumor cells. This disruption in vesicular acidification is likely responsible for the basal inhibition of autophagic flux observed in our study, which we
further characterized by the accumulation of LC3 and LAMP-1 positive punctate co-localizing together that likely represent a population of autolysosomes with a decreased functional capacity for autophagic degradation. Although bafilomycin A1-induced inhibition of V-ATPase has been shown previously to inhibit autophagosome-lysosome fusion \(^{49}\), and mutants of V\(_0\) subunits of V-ATPase inhibit vacuole fusion in a pH-independent manner \(^1\), our results suggest that basal knockdown of ATP6V0C in the absence of pharmacological inhibition by V-ATPase inhibition has the capacity to inhibit autophagic flux through its inhibition of lysosomal pH in a manner that does not require fusion block.

While vesicular acidification and autophagic flux were significantly inhibited, and LC3-II levels significantly increased following knockdown of ATP6V0C under basal conditions, we did not observe a corresponding increase in basal LAMP-1 levels. Although we observed an increase in LAMP-1 and LC3 co-localization that we attributed to inhibition of autophagic flux, it is possible that residual ATP6V0C molecules persisting after knockdown allowed for an incomplete inhibition of basal autophagy that would preclude LAMP-1-positive lysosomes from accumulating in size and/or number. Alternatively, it is possible that basal ATP6V0C deficiency disrupts the participation of V-ATPase in a recently identified, complex signaling network involving mTORC1 and TFEB (transcription factor EB) \(^{30,52}\). TFEB is considered a master regulator for the transcription of lysosome-and autophagy associated genes \(^{36}\). Thus a disruption in TFEB signaling could also explain why basal LAMP-1 levels were not increased following knockdown of ATP6V0C, a possibility that is worthy of future investigation.

The increase in basal levels of α-synuclein and APP observed following knockdown suggests a potential contribution of ATP6V0C and V-ATPase to the onset and progression of age-related neurodegenerative disease. To date, alterations in ATP6V0C have yet to be reported
in human neurodegenerative disease. A previous study has indicated increased expression of the E3 ubiquitin ligase protein RNF182 in Alzheimer’s disease brain. RNF182 was shown using \emph{in vitro} studies to bind ATP6V0C with high affinity and target it for degradation, thus suggesting the potential for disruption of ATP6V0C function in Alzheimer’s disease. As such, future investigation of ATP6V0C expression patterns in brains of neurodegenerative disease patients is warranted. Even if ATP6V0C expression patterns are found to be unaltered in human neurodegenerative disease, recent studies of ATP6V0C over-expression suggest the potential for its therapeutic benefit. Over-expression of ATP6V0C in mouse substantia nigra was shown recently to attenuate behavioral deficits following treatment with a dopaminergic neurotoxin, suggesting its potential utility as a therapeutic target for preventing neurodegeneration.

Furthermore, over-expression of ATP6V0C has been shown \emph{in vitro} to induce HIF-1α gene expression in a pH-independent manner providing further proof of principle that over-expression of one subunit of V-ATPase has functional consequence.

In several of our experimental endpoints, knockdown of ATP6V0C consistently and significantly enhanced the sensitivity of cells to low concentrations of bafilomycin A1. Levels of LAMP-1, LC3-II, α-synuclein and APP CTFs were all significantly higher, and neurite lengths shorter in knockdown cells following treatment with 1 nM bafilomycin A1, a concentration we have shown previously to have little if any influence on autophagy-associated markers or vesicular pH in non-transfected cells, and induce protection against chloroquine-induced death and accumulation of autophagic substrates. ATP6V0C knockdown also caused a significant further reduction in neurite length and induction of cell death following treatment with 3 nM bafilomycin A1. The most plausible explanation for these findings is the incomplete nature of ATP6V0C knockdown, with a resultant decrease in residual binding sites causing a
shift in bafilomycin A1 concentration responsiveness that would allow lower concentrations of bafilomycin A1 to effectively inhibit V-ATPase and in turn inhibit autophagic degradation and induce cell death. It would be interesting for future studies to determine if knockdown of ATP6V0C prevents the ability of low concentrations of bafilomycin A1 to attenuate ALP-associated dysfunction, as we have shown previously in our laboratory. 

Although we observed an approximate 95% reduction in ATP6V0C mRNA, it is possible that heightened sensitivity of knockdown cells to bafilomycin A1 could be explained by residual pools of ATP6V0C protein that were translated prior to knockdown. Our laboratory has tried to detect ATP6V0C protein using several commercially available antibodies and to date we have been unable to reliably detect its protein levels, thus limiting our current assessment of ATP6V0C knockdown to its mRNA levels. Alternatively, these results may be explained in part by the existence of different bafilomycin A1 binding sites within V-ATPase, such as ATP6V0A. Isoforms of ATP6V0A have been shown previously to regulate V-ATPase-dependent $H^+$ transport and are sensitive in yeast to bafilomycin A1-dependent inhibition. Future study of ATP6V0A could provide meaningful insight with respect to the V-ATPase subunit specificity for bafilomycin A1 and its derivatives for regulating ALP-associated degradation.

While 1 nM bafilomycin A1 did not enhance cell death in ATP6V0C knockdown cells, its ability to shorten neurite length suggest its cytotoxic potential that could have resulted in cell death had measurements been taken at later time points. As bafilomycin A1 inhibits V-ATPase completely at 10 nM, it is understandable why treatment of knockdown cells with a ~three-fold lower concentration of bafilomycin A1 (i.e. 3 nM) shown previously to partially inhibit V-ATPase would be more effective than a ten-fold lower concentration (i.e. 1 nM) in causing neurite shortening and inducing cell death. Timing may also explain why an enhanced
knockdown effect was not observed with higher concentrations of bafilomycin A1 (10-100 nM). Had measurements of autophagy substrates and cytotoxicity following treatment with “high” concentrations of bafilomycin A1 been made at time points preceding 48h, the possibility exists for an unmasking of knockdown-specific effects at these concentrations.

The only knockdown-specific alteration for ATP6V0C observed in the present study following treatment with "high, toxic" concentrations of bafilomycin A1 was a significant decrease in α-synuclein monomeric species. This result is difficult to interpret, especially considering that α-synuclein high MW species did not concomitantly increase in knockdown cells following treatment with 100 nM bafilomycin A1. This result may be explained by an enhancement of ubiquitin proteasome system (UPS) function as a consequence to inhibition of the ALP that would in turn enhance degradation of α-synuclein monomer. In support of this argument, cross-talk and compensation of the ALP or the UPS has been demonstrated recently for the degradation of α-synuclein following separate inhibition of either pathway. Interestingly, treatment with a cytotoxic concentration of bafilomycin A1 has been shown recently to inhibit the formation of α-synuclein aggregates, which could explain why the bafilomycin A1 concentration dependence for formation of α-synuclein high MW species in our study was not more robust. However, caution must be used in comparing results between these two studies, as our study assessed levels of endogenous α-synuclein levels and theirs assessed α-synuclein aggregation following over-expression, as it has been shown recently how relative levels of α-synuclein can influence the functional capacity of its degradation by the ALP and the UPS.

In summary, results of the present knockdown study in neuronal cells indicate a contribution of ATP6V0C towards the basal vs. stress-induced function of the ALP and ALP-
associated substrate degradation. Future studies are warranted to validate the utility of ATP6V0C as a therapeutic target to enhance substrate degradation in age-related neurodegenerative disease.

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**Figure 1. Functional validation of ATP6V0C knockdown.** Vesicular acidification following siRNA-mediated knockdown of ATP6V0C was assessed using Lysotracker Red® (LTR). LTR-positive punctae were imaged in differentiated SH-SY5Y cells at 96h following nucleofection of either Non-target siRNA control (A-C) or ATP6V0C siRNA (D-F). DMSO vehicle or 100 nM bafilomycin A1 (BafA1) was added to cells at 3h prior to the addition of LTR. Scale bar = 20 µm. Inset boxes in panels A and D are magnified in panels B and E respectively, with arrows.
indicating LTR-positive punctae. (G) Flow cytometric quantification of LTR relative mean fluorescence intensity (MFI) in vehicle-treated cells at 96h following nucleofection with Non-target vs. ATP6V0C siRNA. Data are expressed as mean ± SEM and are represented by six independent experiments. *p<0.05 vs. Non-target siRNA using one sample t-test.

**FIGURE 2**

**Figure 2. ATP6V0C regulation of autophagy-lysosome pathway markers.** Representative western blots for lysosome marker LAMP-1 (A) or autophagosome marker LC3-II (B) from lysates collected following nucleofection with Non-target or ATP6V0C siRNA and subsequent
treatment for 48h with 0-100 nM bafilomycin A1 (BafA1). Blots were stripped and re-probed for actin (42 kDa) to normalize for gel loading. Data from at least six independent experiments are presented graphically in panels C-F. Within group comparisons of BafA1 concentration responsiveness (Non-target siRNA, open columns, left; ATP6V0C siRNA, filled columns, right) were determined for LC3-II (C) or LAMP-1 (D) by expressing mean ± SEM band intensities relative to actin loading control. All lines above columns indicate significant within-group differences with respect to concentration (*p<0.05 using one-way ANOVA and Bonferroni’s post-hoc test). Comparisons between groups (Non-target vs. ATP6V0C siRNA) for LC3-II (E) and LAMP-1 (F) were determined for each concentration of BafA1 by expressing mean ± SEM fold changes for each ATP6V0C siRNA condition relative to its companion Non-target control. #p<0.05 vs. corresponding Non-target siRNA control using one-sample t-test.
Figure 3. ATP6V0C regulates autophagic flux. Representative western blot for autophagosome marker LC3-II (A) to assess autophagic flux by treating in the presence or absence of the lysosome inhibitors (“LI” in panel A and “LYSO INH” in panel B) chloroquine (100 µM) plus leupeptin (200 µM) for the last 2-4h of a 48h time course. Blots were stripped and re-probed for actin (42 kDa) to normalize for gel loading. Data from seven independent
experiments are presented graphically in panel B. Comparisons between groups (Non-target vs. ATP6V0C siRNA) were determined by normalizing bands to actin loading control and expressing as mean ± SEM fold change for each ATP6V0C siRNA condition (filled columns) relative to its companion Non-target control (open columns). # p<0.05 vs. corresponding Non-target siRNA control using one-sample t-test. (C-H) Representative confocal microscopy images of fixed cells following nucleofection with either Non-target siRNA control (C-E) or ATP6V0C siRNA (F-H) and subsequent treatment with DMSO vehicle for 48h. LC3 immunoreactivity (red, C, F) and LAMP-1 (green, D, G) are shown separately and as merged (E, H). Nuclei are stained in panels E and H with bis-benzimide (blue). Co-localization of LC3 and LAMP-1 immunoreactivity (arrows, panels E, H) suggests inhibition of autophagic flux resulting from accumulation of dysfunctional autolysosomes. Scale bar = 50 μm.
Figure 4. ATP6V0C regulates basal and stress-induced metabolism of alpha synuclein.

Representative western blot for α-syn (A) from lysates following nucleofection and subsequent treatment for 48h with 0-100 nM bafilomycin A1 (BafA1), indicating α-syn high molecular
weight (HMW) species (>50 kDa, suggesting multimeric species) and α-syn monomer (~17 kDa). Blots were stripped and re-probed for actin (42 kDa) to normalize for gel loading. Data from at least six independent experiments are presented graphically in panels B-E. Within groups comparisons of BafA1 concentration responsiveness (Non-target siRNA, open columns, left; ATP6V0C siRNA, filled columns, right) were determined for α-syn HMW species (C) or monomer (D) by expressing mean ± SEM band intensities relative to actin loading control (results of one-way ANOVA were not significant, p>0.05). Comparisons between groups (Non-target vs. ATP6V0C siRNA) for α-syn HMW species (D) or monomer (E) were determined for each concentration of BafA1 by expressing mean ± SEM fold changes for each ATP6V0C siRNA condition relative to its companion Non-target control. #p<0.05 vs. corresponding Non-target siRNA control using one-sample t-test.
Figure 5. ATP6V0C regulates basal and stress-induced metabolism of APP. Representative western blot (A) for amyloid precursor protein C-terminal fragments (APP CTFs) from nucleofected cells following 48h treatment with 0-100 nM bafilomycin A1 (BafA1). An antibody was used that recognized both full-length APP (~110 kDa) and APP CTFs (≤ 15 kDa).
Sizes indicated for CTFs are predicted relative to migration of molecular weight marker and correlate to sizes as previously published (please see results section for further information). In addition to a short-exposure (5 min) blot, a long-exposure (2h) blot is shown to indicate APP CTFs in vehicle-treated cells. Blots were stripped and re-probed for actin (42 kDa) to normalize for gel loading. APP CTFs from long-exposure blots quantified from four independent experiments are expressed graphically (B-C). Within groups comparisons of BafA1 concentration responsiveness (Non-target siRNA, open columns, left; ATP6V0C siRNA, filled columns, right) were determined (B) by expressing mean ± SEM band intensities relative to actin loading control. All lines above columns indicate significant within-group differences with respect to concentration (*p<0.05 using one-way ANOVA and Bonferroni’s post-hoc test). Comparisons between groups (Non-target vs. ATP6V0C siRNA) were determined for each concentration of BafA1 by expressing mean ± SEM fold changes for each ATP6V0C siRNA condition relative to its companion Non-target control. #p<0.05 vs. corresponding Non-target siRNA control using one-sample t-test.
Figure 6. ATP6V0C knockdown exhibit enhanced markers of cytotoxicity. Representative confocal microscopy images obtained from differentiated SH-SY5Y cells following nucleofection with Non-target (A-D) or ATP6V0C (E-H) siRNA and subsequent treatment for 48h with 0 (A, E), 1 (B, F), 3 (C, G) or 10 (D, H) nM bafilomycin A1 (BafA1). Arrows indicate neuritic processes. Scale bar = 50 µm. Neurite length (µm) is expressed graphically (I) and represents results (mean ± SEM) from three independent experiments (10 cells per condition in each experiment). Percent cell death (percentage of propidium iodide (PI)-positive cells quantified using flow cytometry) is expressed graphically (J) as mean ± SEM with data obtained
from a total of nine independent experiments. All lines above columns indicate significant within-group differences with respect to concentration (*p<0.05 using one-way ANOVA and Bonferroni’s post-hoc test). Comparisons between groups (Non-target vs. ATP6V0C siRNA) were determined for each concentration of BafA1 using two-sample t-test (#{p}<0.05).
LOW-DOSE BAFILOMYCIN A1-MEDIATED NEUROPROTECTION IS DEPENDENT ON AUTOPHAGY AND REQUIRES THE V-ATPASE SUBUNIT ATP6V0C

by

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The autophagy-lysosome pathway (ALP) is an essential intracellular homeostatic pathway that transports intracellular materials to the lysosome for degradation and cellular reuse. Preservation of ALP function is critical to neuronal cell viability especially in periods of stress. Inhibition of autophagic flux resulting from either inhibited autophagosome formation or inhibited lysosome-dependent degradation, can induce neuronal dysfunction and death associated with neurodegenerative disease. Agents, such as Bafilomycin A1 (BafA1), and other structurally similar plecomacrolide antibiotics are widely reported to inhibit vacuolar ATPase (V-ATPase) and have high affinity for binding to the V-ATPase subunit, ATP6V0C. BafA1, when used at ≥10 nM, has been shown to cause lysosome dysfunction with autophagosome accumulation and cell death. Similarly, chloroquine (CQ) is a lysosomotropic agent that accumulates in lysosomes causing an elevation in lysosome pH leading to lysosome dysfunction and cell death.

Our lab has previously demonstrated that low concentrations of BafA1 (≤1nM), which do not inhibit V-ATPase, significantly attenuate CQ-induced death of cerebellar granule neurons and differentiated SH-SY5Y neuroblastoma cells. BafA1-mediated neuroprotection was likely regulated through a mechanism that improves autophagic flux. Furthermore, a bafilomycin derivative significantly and dose-dependently attenuated dopaminergic neuron death in C. elegans caused by the overexpression a neurotoxic protein dependent on ALP-mediated degradation. Our current study tested the hypothesis that low-dose BafA1 neuroprotection is dependent on autophagy and requires ATP6V0C. We report that ATP6V0C knockdown, in addition to elevation of lysosome pH, inhibited the protective effects of low-dose BafA1 in our in vitro neuronal model of CQ-induced lysosome dysfunction. We also found that low-dose BafA1-mediated neuroprotection was dependent on autophagy, and was ameliorated in cells...
nucleofected with Atg7 siRNA, which inhibits autophagy at the point of autophagosome formation. Finally, we observed low-dose BafA1 rescued cells from CQ-induced degradation block, an effect that was not seen after ATP6V0C knockdown. Together, our findings suggest that low-dose BafA1 confers neuroprotection through a mechanism that affects both upstream and downstream ALP modulators. Low-dose BafA1 and other therapies that enhance autophagic flux in periods of stress have great therapeutic potential in neurodegenerative pathologies that involve ALP dysfunction and cell death.
INTRODUCTION

The autophagy-lysosome pathway (ALP) is an essential intracellular homeostatic pathway that mediates the delivery of intracellular macromolecules to the lysosome for degradation. The ALP consists of many sub-types of autophagy, including microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is characterized by double-membraned autophagosomes that form around bulk cytoplasm and organelles that are ultimately degraded by the lysosomes. There are conjugation pathways in macroautophagy that form the Atg5-Atg12-Atg16 complex and convert LC3-I to LC3-II, which are activated upstream by Atg7. These conjugation reactions form the autophagosome double membrane. Inhibition of Atg7 prevents these pathways from forming new autophagosome s and as a result macroautophagy is impaired at an early stage. The accumulation of autophagosome s is a sign of intracellular stress, which may result from either enhanced ALP induction or the inhibition of efficient autophagosome clearance, often secondary to lysosome dysfunction. Drugs that disrupt lysosome function, such as chloroquine (CQ) and Bafilomycin A1 (BafA1) are widely used as agents to study ALP dysfunction. Treatment with these agents has been shown by us and others to promote the accumulation of autophagosome s as well as other ALP substrates.

CQ is a widely-prescribed antimalarial agent and has also been used to treat autoimmune diseases. CQ is attracted to acidic environments as a weak base and can move freely across the lysosome membrane. However, upon arrival in the lysosomal lumen, CQ molecules are trapped by the addition of a proton, which results in CQ accumulation and elevated lysosomal pH. CQ-induced death has been described in many cell types including immature neurons and neuronal cell lines in combination with robust accumulation of autophagosomes and ALP substrates.
BafA1 is a macrolide antibiotic that was characterized initially for its selective inhibition of vacuolar-type ATPase (V-ATPase), a two domain complex that is involved in sensing and altering lysosome pH. The peripheral V$_1$ domain hydrolyses ATP to drive the rotary action of the pore-like V$_0$ domain, allowing for proton translocation across the membrane. BafA1 and similarly structured “bafilomycins” are widely used to inhibit the V-ATPase through their interaction with ATP6V0C causing lysosome dysfunction and has been shown to cause neuronal cell death. Nanomolar concentrations of BafA1 are known to disrupt the vesicular proton gradient and increase the pH of acidic vesicles. This disruption of vesicular acidification by both BafA1 and CQ has been proposed to prevent the fusion of autophagosomes. High concentrations of BafA1 are known to inhibit V-ATPase completely, induce vacuolar deacidification, and promote apoptosis.

Our lab has previously demonstrated that low concentrations of BafA1 (≤1nM), which do not inhibit V-ATPase, significantly attenuate CQ-induced death of cerebellar granule neurons and differentiated SH-SY5Y neuroblastoma cells. BafA1-mediated protection is optimal at low concentrations (≤1 nM) which do not inhibit V-ATPase or induce AV accumulation. These data suggest that low-dose BafA1-mediated neuroprotection is independent of its inhibition of V-ATPase. This protection attenuates the accumulation of putatively toxic proteins and associated cell death linked with neurodegenerative disease. However, the mechanisms by which LDB protects the cell from CQ-induced lysosome dysfunction is still unclear.

The role of ATP6V0C in low-dose BafA1-mediated protection has not yet been fully characterized. In one of our recent studies, ATP6V0C knockdown sensitized neuronal cells to BafA1 treatment thereby lowering the concentration of BafA1 necessary to promote toxicity. ATP6V0C knockdown also caused an increase in toxic protein species associated with
neurodegenerative disease. Taken together it is apparent that ATP6V0C provides a prosurvival function in the maintenance of cellular pH and its inhibition is associated with decreased survival in the presence of additional stress.

While these studies provide strong evidence for BafA1-mediated cytoprotection, the mechanism by which low-dose BafA1 modulates ALP function and maintains cell viability has not been investigated. To determine if low-dose BafA1-mediated protection is dependent on ATP6V0C and/or ALP function we genetically manipulated a differentiated neuronal cell line and measured effects on viability and the ALP. We report that low-dose BafA1 confers protection that is dependent on functional autophagy and preservation of lysosome function.

MATERIALS AND METHODS

Cell Culture

Naïve SH-SY5Y human neuroblastoma cells (ATCC, CRL-2266) were maintained in T-75 flasks (Corning, 430641) at 37°C and 5% CO₂ in Minimum Essential Media (Cellgro, 10-010-CV) and F12-K media (ATCC, 30-2004) supplemented with 0.5% sodium pyruvate (Cellgro, 25-000-CI), 0.5% non-essential amino acids (Cellgro, 25-025-CI), 1% penicillin/streptomycin (Invitrogen, 15140-122), and 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific, SH30109.03). Naïve cells received full media change every two days. Prior to their use in experiments, naïve SH-SY5Y cells were differentiated for seven days to a post-mitotic state in 10% FBS media supplemented with 10 μM retinoic acid (RA; Sigma, R2625). Complete RA-supplemented media was replenished every two days.
Nucleofection with siRNA

Small-interfering RNA (siRNA) specific for human ATP6V0C was obtained from Thermo Scientific. The Amaxa Nucleofector™ system (Lonza, VVCA-1003), a modified electroporation system, was utilized to transiently knock down ATP6V0C. First, RA-differentiated SH-SY5Y cells were re-suspended in the Lonza™ Nucleofector reagent. Next, 400nM of re-suspended ATP6V0C siRNA (Thermo Scientific, M-017620-02-0010), or non-target siRNA (Thermo Scientific, D-00126-13-05), (in siRNA buffer with 20 mM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM MgCl₂) was added to the cell suspension. After electroporation, 500 μL of FBS-containing media was immediately added to the cell suspension. Following a 10 min recovery period, nucleofected cells were transferred to T-75 flasks and incubated overnight in 10% FBS differentiation media for 24h. The next day nucleofected cells were plated for experiments (24h after nucleofection and 24h prior to drug treatment) in either eight well glass chamber slides (LabTek, 154941), six well plates (Corning 3516), 48 well plates (Corning ) or 60 mm dishes (Corning, 430166) at a density of 500/mm² in 0% FBS differentiation media containing 2% B-27 supplement (Invitrogen, 17504044). All experimental endpoints ended at either 72h or 96h after nucleofection.

Quantitative Real Time PCR

Cells nucleofected with ATP6V0C siRNA or non-target siRNA were processed for total RNA extraction with the use of TRIzol reagent (Invitrogen, 15596-026). cDNA was generated with oligo (dT) from four µg of RNA using the SuperScript III First Strand Synthesis System (Invitrogen #18080-051). Primers used for detection of ATP6V0C mRNA were ATP6V0C 5’-ATGTCCGAGTCCAAGAGCGGC-3’ and ATP6V0C 5’-CTACTTTTGTGGAGAGGATGAG-
3’. Primers for actin were 5’-GCTCGTCGTCGACAACGGCTC-3’ and 5’-CAAACATGATCTGGTCATCTTCTC-3’. Template for cDNA (2 µL) was added along with 1X Fast SYBR Green Master Mix (ABI #4385610) and water to a final volume of 20µL/well. Assays were performed in triplicate on a Bio-Rad CFX96 instrument using the following conditions: (95°C for three minutes followed by 95°C for 15 seconds and 60°C for one minute for 45 cycles. The comparative cycle threshold (Ct) method (ddCT; 5) was used to determine expression of ATP6V0C in each sample relative to endogenous actin control, which was then used to determine knockdown in ATP6V0C siRNA samples as a percentage of non-target siRNA control.

**Measurement of Acidic Vesicle pH**

After treatment for 48h with DMSO vehicle, cells plated in 60 mm dishes were incubated with LysoTracker Red® (LTR; Life Technologies™, L7528; 100 nM final concentration) for 30 min in Locke’s buffer (15mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1.3mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 1.009 g/L glucose, pH 7.4). Cells were then harvested, pelleted, and re-suspended in 1 X PBS then passed through a 70 µm nylon cell strainer (BD Falcon, 08-771-2) and into collection tubes to provide a single cell suspension. Cells were kept on ice and protected from light during immediate transport to the Joint UAB Flow Cytometry Facility (Enid Keyser, Director) for analysis. A total of 1 × 10⁴ events were detected in each experimental condition using the BD LSR II flow cytometer (Becton Dickinson). Further analysis was performed using FlowJo software (licensed by UAB) to assess mean fluorescence intensity. LTR fluorescence was also visualized via microscopy in cells plated in eight well glass chamber slides and treated for either 48h with DMSO vehicle or with 100 nM bafilomycin A1 for the last 3h of the 48h time
course. Following treatment with LTR, fluorescent images were captured using a Zeiss Axioskop fluorescent microscope (Carl Zeiss Micro-imaging, LLC) at 40X magnification. Transmitted light images were also taken using phase contrast optics.

**Treatment with Bafilomycin A1 and Chloroquine**

Following nucleofection, recovery and plating for experiments (i.e. 48h after nucleofection), media was exchanged for fresh 0% FBS differentiation media containing 2% B-27 supplement in and treated as follows: 0 nM (control), 1, 10, 100 nM BafA1 (AG Scientific, B-1183), 50 μM CQ (Sigma) in the presence or absence of 1 nM BafA1. CQ was prepared at 5 mM stock solutions in 0% FBS media and stored at -20°C. BafA1 was prepared at 10 mM stock solutions in DMSO and stored at -20°C. All cells were maintained at 37°C and 5% CO₂ throughout this protocol. Bafilomycin A1 was prepared as a 10 mM stock solution in DMSO (Sigma, D8418-1L) and stored at -20°C. Unless otherwise noted, all bafilomycin A1 treatments were for 48h, with experiments ending 96h after initial nucleofection.

**Western Blot Analysis**

After treatment for 48h, nucleofected cells in 60 mm dishes were incubated for seven minutes at 37°C with Accutase (Innovative Cell Tech., AT104) to detach cells from their substrate. Whole cell lysates were collected and protein concentrations were determined using BCA protein assay (Fisher Scientific, PI-23227) as previously described. Equal amounts of each protein sample were then electrophoresed using 12% Tris/Glycine SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, 162-0177). Following a 30 minute incubation at room temperature with 5% blocking milk (Bio-Rad, 170-
6404) in 1X tris-buffered saline with tween (TBST), membranes were incubated overnight with primary antibodies for immunodetection of the Atg7 (rabbit anti-Atg7, Cell Signaling). Membranes were washed with 1X TBST containing 0.1% Tween 20 and then incubated with secondary IgG-HRP conjugated antibody for 1h at room temperature. After washing blots, Enhanced chemiluminescence (ECL; Thermo Scientific, PI-32106) was used to detect Atg7. Actin (Sigma, A1978) was used to normalize for gel loading. Films with detected bands of interest were scanned using Adobe® Photoshop® and band intensities were calculated using UN-SCAN-IT gel digitizing software (Silk Scientific, Inc.).

**Measurement of Cell Viability**

Viability of SH-SY5Y cells was determined after 48h treatment using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric reduction assay. MTT stock solution was prepared using sterile PBS (2mg/mL). 50 μL of MTT stock solution was then added to each well containing 200 uL cells plus media (final volume = 250 μL or .4 mg/mL) and incubated for 2h at 37°C and 5% CO2. Note: MTT stock solution was added to a blank well containing 200 μL media for subtracting background values. Following the initial incubation, MTT plus media solution was carefully aspirated from each well, including the blank well noted above, and replaced with 200 μL of DMSO and incubated for 30 minutes at 37°C. Individual treatment groups will be quantified at a wavelength of 570 nm using a spectrophotometer.
Assessment of Autophagosome-Lysosome Fusion

Following nucleofection with ATP6V0C siRNA or non-target control siRNA, cells plated in eight well glass chamber slides were treated for 48h with DMSO and subsequently fixed for 15 min at 4°C using Bouin’s fixative (75% saturated picric acid, 23.8% of a 37% w/v formaldehyde solution, 4.7% glacial acetic acid) in the presence of 0.5% saponin (Sigma, 47036). Following incubation for 30 min with 1X PBS blocking buffer containing 1% bovine serum albumin, 0.2% non-fat dry milk and 0.3% Triton X-100, cells were incubated overnight at 4°C in blocking buffer without Triton X-100 and containing rabbit anti-LC3 antibody (Sigma, L7543) to detect autophagosome punctae. Following PBS wash, fixed cells were incubated for 1h with SuperPicTure™ (Invitrogen, 879263) anti-rabbit IgG secondary antibody. Following PBS wash, fixed cells were fixed cells were subjected to tyramide signal amplification (incubation for 30 min with Cy3 plus tyramide, Perkin Elmer, FP1170) to detect LC3. Next, fixed cells were incubated for 10 min with 3% hydrogen peroxide to neutralize residual peroxidase from the first secondary antibody. After PBS wash and 30 min incubation with blocking buffer, fixed cells were incubated overnight at 4°C in blocking buffer containing the second primary antibody, mouse anti-human LAMP-1 (University of Iowa Hybridoma Bank, H4A3) to detect lysosomes. Following subsequent PBS wash, fixed cells were incubated for 1h with Vector Impress (Vector Labs, MP-7402) anti-mouse IgG. Following PBS wash, tyramide signal amplification was performed (incubation for 30 min with FITC plus tyramide, Perkin Elmer, FP1168) to label LAMP-1. Following PBS wash, fixed cells were last incubated with bis-benzimid (0.2 µg/ml in PBS, Sigma) to label nuclei. Images were captured using a Zeiss Observer.Z1 laser scanning microscope equipped with a Zeiss 40X Plan-Apochromat objective and imaged using Zen 2008 LSM 710, V5.0 SP1.1 software.
**Statistical Analysis and Figure Preparation**

Quantitative real time PCR data were expressed as mean ± SEM percent knockdown for ATP6V0C. LTR staining was presented as mean ± SEM fluorescence intensity. For assays of viability (MTT reduction), values within a single experiment will be generated by averaging replicates from at least three different wells and will be expressed as percent non-transfected/non-target/vector control for any given treatment.

Western blot band intensities Atg7 was normalized to actin loading control, and was expressed (mean ± SEM) either as such to assess the effects of Atg7 siRNA. Neurite length was presented as mean ± SEM average length (µm) per cell. To determine effects of genetic manipulation, between groups comparisons of Non-target vs. ATP6V0C siRNA were evaluated for significance using either one-sample t-test (LTR data; western blot data normalized as fold change relative to the companion non-target control) or two-sample t-test (assessment of neurite length and percent cell death). For all tests, statistical significance was set *a priori* at *p* < 0.05. Graph Pad Prism® was used to perform statistical analysis and generate graphs, and Adobe® Photoshop® was used to assemble figures. A minimum of three independent replicates were performed for each experimental endpoint.

**RESULTS**

**Validation of ATP6V0C knockdown**

We measured siRNA mediated knockdown of ATP6V0C using quantitative real time PCR analysis on cDNA generated from RNA in samples isolated from differentiated SH-SY5Y human neuroblastoma cells harvested 96h following nucleofection with non-target or ATP6V0C siRNA. Using the comparative cycle threshold method (Ct) with actin mRNA acting as an
internal standard. We found that ATP6V0C message was reduced significantly with only 5.6% remaining when compared to non-target controls (Fig. 1A). Next we tested lysosome acidity by LTR as a measure of functional alterations after ATP6V0C knockdown. LTR is attracted to acidic cellular compartments and will accumulate in a pH dependent manner that is observed as an intense red fluorescence in acidic environments that will decrease as pH increase. We found that ATP6V0C knockdown markedly attenuated LTR fluorescence, suggesting ATP6V0C regulates lysosome pH (Fig. 1B).

**Low-Dose BafA1-mediated neuroprotection requires ATP6V0C**

Cell viability was measured by MTT assay 96h post-nucleofection and 48h after treatment with BafA1 (1-100nM), CQ (50 µM), or both. ATP6V0C knockdown causes sensitivity to BafA1 (1-10nM) that results in a significant reduction in cell viability (Fig 2, \( p^* \leq 0.05 \)). There are no ATP6V0C-depedent effects on cell viability at either the highest concentration of BafA1 (100nM) or with CQ treatment (50µM). However, when non-target control cells were treated with both CQ and low-dose BafA1 (1nM) we observed the preservation of cell viability when compared to CQ treatment alone (Fig 2, \( p^{**} \leq 0.05 \)). This protective effect was attenuated and cell viability was not significantly different from CQ treatment in cells nucleofected with ATP6V0C siRNA (Fig 2, n.s.).

**Low-Dose BafA1-Mediated Neuroprotection Is Dependent On Autophagy**

First we confirmed siRNA-mediated knockdown of Atg7 at 96h post-nucleofection. Atg7 protein levels were reduced compared to their non-target scrambled controls in both nutrient rich and depleted conditions (Fig. 3A). We quantified this knockdown caused by Atg7 siRNA to be
37.4% of the amount found in non-target control cells in complete media (Fig. 3B). Knockdown of Atg7 almost completely ameliorated the detection of LC3-II conversion, an indicator of autophagosome levels, in both complete media and starvation media (Fig. 3A). Finally we tested the effects of Atg7 knockdown on low-dose BafA1-mediated neuroprotection against CQ-induced lysosome dysfunction and found that protection was lost cells with knockdown when compared to their non-target control (Fig. 3C). This suggests that the ALP must function uninhibited to maintain low-dose BafA1-mediated neuroprotection.

**Autophagic Flux is Enhanced by Low-Dose Bafa1 in a ATP6V0C-Dependent Manner**

To determine the effects of low-dose BafA1 treatment on autophagic flux we used immunocytochemistry techniques to label the autophagosome marker LC3 (cy3 red) and the lysosome membrane marker LAMP-1 (FITC green) and observed their interaction in cells nucleofected with either non-target siRNA or ATP6V0C siRNA. We found that there no noticeable co-localization (seen as yellow) in untreated cells (Fig. 4A, 4B). However, in ATP6V0C knockdown cells we see more co-localization of LC3 and LAMP-1 signal (Fig. 4D) when compared to their non-target control (Fig. 4C), suggesting decreased lysosome degradation. CQ induced robust co-localization of LC3 and LAMP-1 indicating strong inhibition of autophagic flux, but no ATP6V0C-dependent differences were detected (Fig. 4E, 4F). In non-target control cells we observed an attenuation of CQ-induced co-localization of LC3 and LAMP-1 (Fig. 4G) indicating improved autophagic flux, an effect that is not maintained after knockdown of ATP6V0C (Fig. 4H).
DISCUSSION

The goal of this study was to better elucidate the mechanism by which low-dose BafA1 confers neuroprotection against CQ-induced lysosome dysfunction. We have provided the first evidence that low-dose BafA1 requires ATP6V0C for its reported ability to attenuate CQ-induced neuronal cell death (Fig. 2). Furthermore, we found that inhibition of the ALP by siRNA-mediated knock down of Atg7 attenuates neuroprotection against lysosome dysfunction afforded by low-dose BafA1 (Fig. 3). We found that inhibition of ATP6V0C increased ALP-related sensitivity to BafA1 even at low concentrations and was demonstrated by increased LAMP-1 and LC3 co-localization (Fig. 4), which suggests an inhibition of autophagic flux that likely negated low-dose BafA1’s pro-survival stimuli in the presence of CQ.

Our siRNA-mediated knockdown significantly reduced ATP6V0C mRNA levels (Fig 1a). Thus, to date our lab has not been able validate the utility of an antibody that can identify ATP6V0C by western blot. As such it is not clear how much residual ATP6V0C is present during the course of our experiments. However, we have previously demonstrated consistent microscopic changes associated with ATP6V0C knockdown including a decrease in neurite length and a decrease in lysosome acidification (Fig 1b), both of which likely contribute to the deleterious consequences of knocking down ATP6V0C. Lysosome acidification is also inhibited, although not to the degree that is seen with 100nM BafA1 treatment for 3-4h (data not shown), thus suggesting that partial acidification remains.

Low-dose BafA1-mediated protection against lysosome dysfunction was attenuated by nucleofecting cells with siRNA targeting ATP6V0C. ATP6V0C knockdown causes sensitivity to BafA1 after knockdown of ATP6V0C that closely resembles the increased toxic effects of anti-cancer drugs on cancer cells previously unresponsive to treatment (REF). In agreement with our
previous data (pg. 89) we report that knockdown of ATP6V0C reduces cell viability following treatment with 1nM BafA1 when compared to non-target control cells (Fig. 2). The V0 proteolipid ring is made of different subunits that closely resemble each other in structure and function, and are known to exhibit conformational changes that can affect lysosome acidification and V-ATPase assembly. There is evidence in the literature that BafA1 may potentially bind other sites thus it is possible that knockdown of ATP6V0C causes conformational changes in the V0 proteolipid ring that could expose other potential binding sites.

When we tested the effects of ATP6V0C knockdown on low-dose BafA1-mediated protection against CQ-induced toxicity we found, as expected, loss of protection. The potential mechanism by which ATP6V0C mediates low-dose BafA1 protection against CQ-induced toxicity remains unclear. However, in combination with our previously data, which indicates that low-dose BafA1 attenuates markers of ALP dysfunction (AV accumulation; inhibition of autophagic flux; increase in α-synuclein) it would be logical to predict that low-dose BafA1 confers protection through its enhancement of ALP function. While loss of protection may be a caused, in part, by lysosome dysfunction, the possibility that BafA1 could be exerting its pro-survival effects by some alternate mechanism cannot be ruled out. There is evidence that BafA1 and ATP6V0C mediate transcription through interaction with hypoxia inducible factor 1(HIF-1α), a transcription factor that is known to participate in cell survival functions. Other studies supporting a cytoprotective role for ATP6V0C include the report that its expression is enhanced in cancer cells that are resistant to chemotherapeutic-induced death, and that its RNAi-mediated knockdown in cancer cells exacerbates stimulus-induced cell death. Furthermore, in the context of neurodegenerative disease ATP6V0C over-expression in vivo was able to regulate neurotransmission and improve results on behavioral tests. The direct contribution of ATP6V0C
to cellular viability is unclear however these studies demonstrate that it does provide potentially numerous avenues for further exploration. And so, we are also interested in the potential for over-expression of ATP6V0C as a method of inducing low-dose BafA1-like effects on ALP function that also increase cell viability, without the potential for toxicity that is associated with BafA1.

To determine if low-dose BafA1-mediated protection requires the ALP we conducted siRNA-mediated knockdown of Atg7 to prevent autophagosome formation. We specifically focused on Atg7, a known mediator of both conjugation pathways necessary for autophagosome formation, to determine if basal ALP activity is critical to low-dose BafA1 protection against lysosome dysfunction. The downstream effects of Atg7 inhibition were seen functionally in reduced levels of LC3-II conversion (Fig. 3), which indicates decreased autophagosome formation. Our previously published data indicate that ALP enhancement after low-dose BafA1 treatment effectively blocks the deleterious effects of CQ, however in this present study we found that low-dose BafA1 requires the uninhibited formation of autophagosomes to confer its protective effects (Fig. 3). After independent 48h treatments with BafA1 and CQ there was no evidence of Atg7-dependent alterations in neuronal toxicity, however after 48h dual treatment any protection afforded by low-dose BafA1 was lost in cells that had undergone Atg7 knockdown. This result suggests the ALP is necessary, specifically in the formation of autophagosomes, for the protection against lysosome dysfunction that is conferred by low-dose BafA1. This result provides proof of principle that low-dose BafA1 requires the ALP to reduce cellular toxicity in periods of stress, specifically.

Additionally we used immunocytochemical detection methods and confocal microscopy to determine if low-dose BafA1 exhibited ATP6V0C-dependent alterations in autophagic flux.
Co-localization of LC3 and LAMP-1 was decreased in non-target cells following treatment with low-dose BafA1 and CQ when compared to the ATP6V0C knockdown cells, suggesting inhibition of autophagic flux. We demonstrate no ATP6V0C-dependent interaction of LC3 and LAMP1 under basal conditions. However, after ATP6V0C knockdown and treatment with 1nM BafA1 there was qualitatively more colocalization of LC3 and LAMP-1, an effect that is interpreted as a block in autophagic flux. Combined treatment with low-dose BafA1 and CQ reduced LAMP-1 detection in the non-target controls compared to ATP6V0C knockdown cells suggesting ATP6V0C maintains autophagic flux in a way that confers improved viability. Increased LAMP-1 and LC3 colocalization is seen in neuroblastoma cells and has also been correlated with lysosome dysfunction prior to cell death. Agents such as rapamycin (mTOR-dependent) and trehalose (mTOR-independent) are known to induce autophagy have been used effectively to restore ALP function leading to improved cell viability in models of neurodegeneration.

Additionally, it would be of particular utility to further determine the effects of low-dose BafA1 on upstream autophagy signaling regulators such as mTOR and its downstream targets, which may participate in neuronal survival. mTOR and V-ATPase have been recently linked to interact as modulators of autophagic signalling and rely on transcription factor EB (TFEB) to control a network of genes which regulate autophagy and lysosome biogenesis. TFEB is inhibited by mTOR, but under conditions of lysosome dysfunction TFEB is translocated to the nucleus through inhibition of mTOR, an effect that can be induced by rapamycin. If the low-dose BafA1 effect is shown to be transcriptionally regulated it would open the door to therapies that have similar effects on the upstream signaling pathways that promote autophagic flux but without the potential for toxicity through use of BafA1.
Neurite length, as an indicator of neuronal structural stability, after ATP6V0C knockdown has been shown to be reduced under basal conditions and BafA1 treatment (1nM, 3nM) when compared to appropriate non-target controls (pg. 89). Neurites are particularly dependent on efficient ALP-mediated degradation as they rely on retrograde transport of autophagic cargo to maintain their synaptic integrity. Accumulation of autophagosomes within neurites is reported to contribute to synaptic dysfunction and degradation. Because we observed shortened neurites after ATP6V0C knockdown, we predict that low-dose BafA1 would not likely preserve neuritic structure without basal levels of ATP6V0C. We are presently conducting experiments that focus on ALP dysfunction and its promotion of neurite retraction or shortening, and whether or not low-dose BafA1 may preserve neurite length in an ATP6V0C-dependent way as a further indication of its mechanism of protection.

Our data is consistent with the belief that low-dose BafA1-mediated protection is directly linked to maintenance and/or enhancement of ALP function in periods of lysosome dysfunction. Through the elucidation and characterization of the actual mechanism of low-dose BafA1-mediated protection, our lab and others will be able to develop strategies to replicate this protection through methods not normally associated with cellular toxicity.
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Figure 1. Validation of ATP6V0C knockdown. Quantitative real-time PCR was used to assess ATP6V0C mRNA levels (A). Vesicular acidification following siRNA-mediated knockdown of ATP6V0C was assessed using Lysotracker Red® (LTR). LTR-positive punctae were imaged in differentiated SH-SY5Y cells at 96h following nucleofection of either Non-target siRNA control or ATP6V0C (B). Scale bar = 20 µm. #p<0.05 vs. Non-target siRNA using one sample t-test.
Figure 2. ATP6V0C knockdown causes BafA1 sensitivity and inhibits low-dose BafA1-mediated neuroprotection. Percent cell viability (MTT assay) is expressed graphically (Fig. 2) as mean ± SEM with data obtained from at least 9 independent experiments in cells 96h post-nucleofection and 48h after treatment. The line above columns indicate significant within-group differences after low-dose BafA1-mediated neuroprotection (**p<0.05 using one-way ANOVA and Bonferroni’s post-hoc test). Comparisons between groups (Non-target vs. ATP6V0C siRNA) were determined for each concentration of BafA1 using two-sample t-test (#p<0.05).
Figure 3. ATP6V0C regulates basal and stress-induced metabolism of alpha synuclein.

Representative western blot for Atg7 (A) from lysates following nucleofection and subsequent incubation in complete media throughout 48h prior to collection compared with cells incubated in starvation media (PBS) for the final 2h (Fig. 3A). Both nutrient rich and starvation conditions after knockdown displayed reduced levels of LC3-II when compared to non-target controls (Fig. 3A). Blots were stripped and re-probed for actin to normalize for gel loading. Data from 4
independent experiments in complete media after Atg7 knockdown demonstrate significant reduction of Atg7 protein levels (Fig. 3B). Comparison of low-dose BafA1 and CQ treatments alone after knockdown indicated no Atg7 dependent on neuronal viability. However, after knockdown of Atg7, cells treated with CQ and low-dose BafA1 in combination were significantly less viable when compared to their non-target controls (Fig. 3C, *p<0.05 vs. corresponding Non-target siRNA control using 2-sample t-test).

Figure 4. Low-dose BafA1 requires ATP6V0C to enhance autophagic flux. Representative confocal microscopy images of fixed cells following nucleofection with either Non-target siRNA control (A, C, E, G) or ATP6V0C siRNA (B, D, F, H) and subsequent treatment with DMSO vehicle for 48h. LC3 immunoreactivity (red) and LAMP-1 (green) are as merged in all panels as an indicator of autophagic flux. Nuclei are stained with bis-benzimide (blue). There does not appear to be a block in autophagic flux in either set of untreated cells (A, B). Colocalized LC3
and LAMP-1 immunoreactivity (panels D, E, F, H) suggests inhibition of autophagic flux resulting from accumulation of dysfunctional autolysosomes. Decreased co-localization of LC3 and LAMP-1 indicate low-dose BafA1 restores autophagic flux non-target cells (G). Scale bar = 20 μm
DISCUSSION

The aims of our studies were to test the global hypothesis that alterations in the ALP contribute to neuronal dysfunction and death in models of neurodegenerative disease. First we tested the effects of rotenone, a drug known to cause PD-like pathologies, on ALP function to elucidate the mechanism that leads to α-synuclein accumulation. Next, we genetically inhibited ATP6V0C, a V-ATPase membrane associated subunit, by siRNA-mediated knockdown to better its role in the maintenance of ALP function under basal and stressed conditions. Finally we examined the role of ATP6V0C in the protection seen previously with low-dose BafA1 on chloroquine-induced lysosome dysfunction. Our in vitro models utilized pharmacological stressors and genetic manipulation to elucidate the involvement of the ALP in PD-associated pathologies. In developing a better understanding of the ALP’s role in neuronal dysfunction and degeneration we are more able to identify potential therapeutic targets to combat neurodegenerative disease.

Maintenance of acidic lysosomal pH is a necessary homeostatic cellular function that provides an optimal environment for the activity of acidic hydrolases responsible for ALP-mediated degradation. Previous studies provide conflicting data on whether regulation of vesicular pH is dependent on ATP6V0C. Knockdown of ATP6V0C has been previously reported in one study to increase acidic vesicle pH \(^{100}\), while in another it did not alter vesicular pH \(^{13}\). In our study we observed an elevation of acidic vesicle pH after knockdown of ATP6V0C that did not alter neuronal viability but did sensitize the cells to further pharmacological treatment with BafA1. siRNA-mediated inhibition of ATP6V0C
did not have any associated toxicity and it is probable that basal ATP6V0C remained due to the transient nature of our genetic inhibition. However, while we have been unable to detect ATP6V0C at the protein level, our results indicating a functional consequence of ATP6V0C suggest knockdown at the protein level. It will be important in future studies to quantify protein levels following knockdown to more accurately assess degree of knockdown. Although cell death was not increased after knockdown alone, toxicity was dramatically increased when combined with BafA1 treatment (1nM-3nM). This result was due likely to genetic inhibition of ATP6V0C causing increased sensitivity of V-ATPase to inhibition by BafA1. The ATP6V0C binding pocket for BafA1 was found through site-directed mutagenesis, which prevented inhibition of the V-ATPase complex \(^8\). However, it appears that knockdown of ATP6V0C, leading to its absence from the V-ATPase complex, may be promoting its sensitivity to BafA1. The V-ATPase is an essential regulator of lysosome acidification and numerous reports of its inhibition link altered V-ATPase function with cellular dysfunction and decreased viability \(^3, 44, 60\).

Alternatively, these results may be explained in part by the existence of different BafA1 binding sites, such as ATP6V0A. Isoforms of ATP6V0A have been shown previously to regulate V-ATPase-dependent H\(^+\) transport and are sensitive in yeast to bafilomycin-dependent inhibition \(^86\). In addition, other ion transporters at the lysosomal membrane could be modulated to compensate for the inhibition of V-ATPase limiting its deleterious effects, an area of research worthy of further exploration.

It is reported that rotenone inhibits complex I of the mitochondrial electron transport chain inducing toxic levels of oxidative stress and increased ALP induction \(^50, 52\). Rotenone is also implicated in causing lysosome dysfunction, but the mechanism by
which rotenone causes lysosome dysfunction is not entirely clear. Our results suggest that rotenone induces lysosome dysfunction by causing an elevation in pH that occurs through cellular depletion of ATP. Thus, we propose that in periods of low ATP availability the V-ATPase machinery cannot properly acidify the lysosome leading to the accumulation of ALP substrates. To our knowledge this is the first report of rotenone’s effects on lysosome pH in association with depleted ATP levels, suggesting a potential mechanism for inhibiting the proton-motive function of V-ATPase at the lysosomal membrane.

The V-ATPase provides a common intersection where multiple cellular pathways cross, and can be targeted in numerous ways for therapeutic benefit. A recent study concluded that the V-ATPase membrane-integrated V0 domain, the pore-like structure containing ATP6V0C that transports protons across various membranes in regulation of vesicular and cellular pH, also acts as a sensor of vesicular pH that controls exocytosis and synaptic neurotransmission via the reversible dissociation of the V1 domain. We would be particularly interested in the future to determine if BafA1 interaction with the V0 domain could potentially be used as a method to rid the cell of toxic undegraded materials through exocytosis. Furthermore, the V0 domain has also been linked to SNARE-dependent exocytosis where a conformational change in V0 proteolipids are reported to stimulate fusion by creating a hydrophobic crevice that promotes lipid reorientation and formation. The idea of BafA1 regulating V-ATPase assembly and disassembly as a method of inducing vesicle exocytosis was recently bolstered by evidence that BafA1 limited recruitment of the V1 domain, in turn keeping it disassociated from the V0 domain. This could be further explored as a method for the cell to remove vesicular waste that cannot be efficiently degraded intracellularly.
The accumulation of autophagosomes in neuronal cells that is associated with a wide range of neurodegenerative diseases is caused by either elevated autophagic induction or inhibition of lysosome-dependent degradation. Biochemical detection of LC3-II by western blot analysis is considered the gold standard for assessment of autophagosome levels\textsuperscript{41}. Our first evidence of alterations in the ALP was observed as elevated autophagosome accumulation in lysates from differentiated neuronal cells stimulated by either rotenone exposure or siRNA-mediated knockdown of ATP6V0C. Since there were relatively moderate increases autophagosome detection after both stressors, it was logical to predict that there was either a slight elevation in autophagic induction or inhibition of degradation. Our autophagic flux assay did not demonstrate evidence of increased induction in knockdown cells nor those treated with rotenone. However it did suggest that their existed a partial degradation block after both stressors.

Rotenone treatment increased biochemical detection of the ALP substrate p62, an autophagic cargo linker protein that is commonly used to measure ALP-dependent degradation, that when detected at elevated levels indicates decreased lysosome degradation. In a previous study rotenone was shown to induce the accumulation of p62\textsuperscript{31}, an autophagic cargo linker protein that is commonly used to measure of ALP-dependent degradation, while in another it caused a reduction of p62 concomitant with increased detection of autophagosomes\textsuperscript{94}. Although p62 levels were increased in our rotenone model of PD, indicating inhibition of autophagy, it would be interesting to further characterize p62 localization within the cell and its association with other ALP substrates. Colocalization of α-synuclein and p62 has been previously reported in a similar model, suggesting their lysosome-mediated degradation; however their
persistence would likely be due to defective ALP\textsuperscript{87}. If these proteins were found to be colocalized in our hands it would be of particular utility to determine what proportion of aggregates were also colocalized with LC3, which would indicate autophagosome-mediated sequestration but inefficient degradation\textsuperscript{41}. Using our rotenone model we would predict that α-synuclein would likely colocalize with both p62 and LC3 after rotenone treatment due to inefficient degradation resulting from elevated lysosome pH.

Pharmacological methods to enhance autophagy have been used experimentally to prevent rotenone-induced death and may be able to provide protection in periods of lysosome dysfunction\textsuperscript{21,31,66,90}.

Neuronal function is dependent upon neurites that project out from their cell body and form synapses with other cells. Neurite length is dependent on multiple stimuli and reduced neurite length is reported due in part to dysfunctional autophagy\textsuperscript{62}. Furthermore, neurites that do not form synapses or retract from synapses are evidence of neuronal dysfunction and have been shown to accumulate autophagosomes in models of neurodegeneration\textsuperscript{17}. As previously published studies have not investigated the role of V-ATPase, or its subunit ATP6V0C, in the regulation of neurite length we decided to investigate neurite length after knockdown of ATP6V0C. Interestingly we detected a reduction in neurite length that occurs after knockdown of ATP6V0C alone, and sensitizes cells to further BafA1-mediated reduction. The loss of neurite length in our studies is believed to be caused by inhibition of lysosome degradation, accompanied by the accumulation of autophagosomes. Prevention of autophagosome accumulation is reported to preserve neuritic structure\textsuperscript{62}. The decrease in neurite length observed in our hands is in agreement with a recent study of lysosome dysfunction that reported altered
neurite morphology concomitant with inhibition of autophagy and the accumulation of proteins with neurotoxic potential\textsuperscript{48}. Therapies developed to enhance lysosome function and improved the clearance of neurotoxic proteins could be used to effectively preserve the neuritic architecture that is necessary for neuronal homeostasis.

Autophagic machinery and lysosomal biogenesis are coordinated through a gene network that is sensitive to lysosome inhibition\textsuperscript{19}. This gene network is regulated by the transcription factor EB (TFEB), which is phosphorylated (inactivated) by and co-localizes with mTOR on the lysosomal membrane during periods of suppressed autophagy, but is activated and subsequently translocated to the nucleus stimulating transcriptional activation after lysosome disruption or induction of autophagy\textsuperscript{69}. Following rotenone treatment we detected increased lysosomal volume or number by elevation of LAMP-1, a lysosomal membrane protein that is best known for its regulation of lysosomal motility and endosomal-lysosomal fusion with autophagic vacuoles\textsuperscript{36}. This increase in LAMP-1 suggests an increase in either the number or size of acidic vesicles that may be a consequence of lysosome dysfunction\textsuperscript{96}. Conversely, LAMP-1 may also be altered as a function of de novo lysosome biogenesis, as shown recently under stressful conditions and as a response to alterations in autophagy\textsuperscript{19}. To address this possibility the effects of rotenone on endogenous levels of TFEB were assessed as it regulates transcription of LAMP-1 in addition to other lysosomal targets. Levels of TFEB following rotenone treatment were unchanged, suggesting that the increase in LAMP-1 observed following rotenone treatment does not result from lysosome biogenesis, but rather from the inhibition of lysosomal degradation. Agents that promote lysosome dysfunction may also play a role in influencing gene expression of a coordinated network
of lysosomal and autophagy genes. It is important to note that while levels of TFEB may be unchanged the localization of TFEB may be of greater importance as its translocation to the nucleus promotes transcription of lysosome-associated genes. Thus, TFEB could be exogenously expressed and/or activated as a protective response to stress-induced lysosome dysfunction. Genetic manipulation of TFEB in our model may provide a useful complimentary approach in future studies to further characterize the putative role of ATP6V0C in regulating the clearance of ALP substrates.

The aggregation of proteins with neurotoxic potential, such as α-synuclein in PD, is considered a central theme in numerous neurodegenerative diseases and is believed to contribute to the disease pathogenesis. Here we report that α-synuclein high molecular-weight (HMW) species (indicative of α-synuclein aggregation) putatively dependent on the ALP for clearance were increased biochemically after rotenone treatment, and ATP6V0C knockdown under basal or stressed conditions. It is reasonable to predict that the size of these aggregates may preclude proteasome-mediated degradation probably because these aggregates are too large to enter the proteasome barrel. In light of this data, lysosomal degradation becomes even more important to cell viability because aggregated proteins would be preferentially routed to the ALP for degradation. Failure to degrade these aggregates could lead to cellular toxicity that may lead to further ALP dysfunction as α-synuclein has been linked with inhibition of the ALP, at stages of formation and completion.

While our data indicate lysosome dysfunction as a major contributor to α-synuclein accumulation, the causes of decreased lysosome-dependent degradation of autophagosomes and other ALP substrates may be potentially due to defects in their
microtubule-dependent vesicular transport structure or machinery. Deficits in this transport mechanism have been associated with increased autophagosome accumulation, increased p62 detection, and lysosomal fusion block\textsuperscript{49}. Therefore restoration or enhancement of dynein-dependent transport could provide an effective strategy to reduce autophagosome accumulation through improved delivery to the lysosome for degradation\textsuperscript{37}.

The protective effects of low-dose BafA1 on chloroquine-induced lysosome dysfunction were first described by Shacka et al. 2006 by its ability to alleviate dose-dependent inhibition of autophagy, apoptotic signaling, and cell death\textsuperscript{72}. Low-dose BafA1 also attenuated the chloroquine-induced reduction in processing of the mature 'active' form of cathepsin D\textsuperscript{61}, the principal lysosomal aspartic acid protease responsible for the degradation of α-synuclein, suggesting BafA1 inhibition of neuronal cell death is a potentially novel mechanism of action apart from its ability to inhibit V-ATPase. Chloroquine was shown to induce autophagosome accumulation and inhibited autophagic flux, effects that were attenuated upon treatment with low-dose BafA1 and were associated with a significant decrease in chloroquine-induced accumulation α-synuclein aggregates. In addition, a bafilomycin derivative significantly and dose-dependently attenuated dopaminergic neuron death in \textit{C. elegans} resulting from \textit{in vivo} over-expression of human wild-type α-synuclein\textsuperscript{60}. However, it is currently unknown whether BafA1-mediated protection is dependent on the ALP for the execution of its therapeutic effects. Our current study reports that low-dose BafA1-mediated protection against cell death is dependent on the autophagy protein Atg7 and the V-ATPase subunit ATP6V0C. After independent experiments siRNA-mediated knockdown of Atg7 and ATP6V0C we
observed the attenuation of protection afforded by low-dose BafA1 against chloroquine-induced death. ATP6V0C knockdown could be expected to inhibit protection by low-dose BafA1 due to its high affinity and interaction with BafA1. We observed a shift in the dose response of BafA1 that sensitized cells to increased autophagosome accumulation and cell death associated with lysosome dysfunction so we did not expect low-dose BafA1 to provide protection since the ALP was likely already compromised.

Atg7 knockdown-mediated attenuation of low-dose BafA1 protection is more difficult to interpret. Our present data suggests low-dose BafA1 protection is dependent on Atg7, the ALP activator protein necessary for the dual conjugation pathways’ regulation of autophagosome formation. Atg7 knockdown consistently inhibited the detection of LC3-II by western blot in both basal and stressed conditions, confirming that autophagic induction at an early stage of autophagosome formation is inhibited. Knockdown of Atg7 ameliorated low-dose BafA1-mediated protection, suggesting protection is dependent on autophagosome formation. Autophagic flux appears to be restored as indicated by the reduction in colocalization of LC3 and LAMP-1 following treatment with low-dose BafA1. This result is in agreement with our previous data suggesting low-dose BafA1 confers protection by enhancing autophagic flux\textsuperscript{60}. Together, our findings suggest that low-dose bafilomycin is cytoprotective in part through its maintenance of the ALP, and underscores its therapeutic potential for treating PD and other neurodegenerative diseases exhibiting disruption of protein degradation pathways and accumulation of toxic protein species.

Our studies aimed to test the hypothesis that alterations in the ALP cause a block in flux and induce lysosome dysfunction, both of which contribute to neuronal
dysfunction and death in neurodegenerative disease. We have demonstrated that stimuli that increase lysosome pH trigger autophagosome accumulation. There are a multitude of studies that indicate lysosome dysfunction is integral to the pathogenesis in many neurodegenerative diseases. Our studies have shown that low-dose BafA1-mediated improvements in neuronal viability require functional autophagy and basal levels of ATP6V0C. Our manipulation of the ALP by either pharmacological or genetic means has highlighted the importance of autophagic degradation by the lysosome as a homeostatic and protective mechanism that, when compromised, may lead to the promotion of toxic protein aggregation and cellular dysfunction and death.

Current treatment methods for PD include dopamine replacement, dopamine agonists, neuronal precursor transplantation, and by surgical means with deep brain stimulation\textsuperscript{24, 34, 89}. All these methods represent reactive treatments for patients already experiencing symptoms due to robust loss of nigral dopaminergic neurons and subsequent decrease in dopamine neurotransmission. Our studies provide strategies intended to support dopaminergic neuron viability which may provide early stage protection against PD progression. Models using pharmacological intervention as well as gene therapies which elevate ALP function have been shown to promote neuronal survival concomitant with decreased cellular pathology, and provide proof of principle for future potential use in clinical settings. Strategies to improve the efficiency and capacity of the ALP are necessary to preserve neuronal function and viability in the context of PD and other autophagy-related neurodegenerative diseases.
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