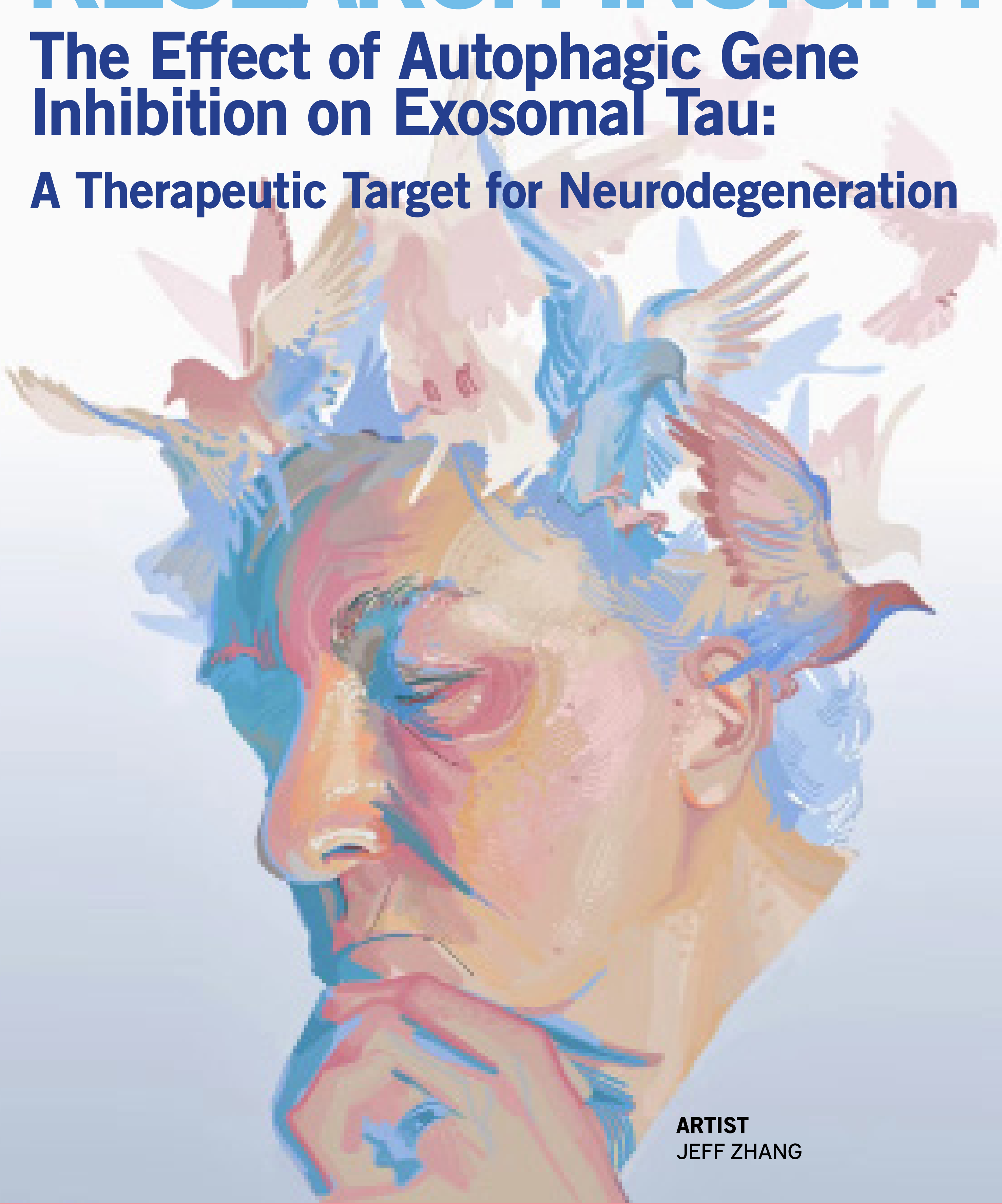
RESEARCH INSIGHT

The Effect of Autophagic Gene Inhibition on Exosomal Tau:

A Therapeutic Target for Neurodegeneration



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ABSTRACT

Philip Yu is an undergraduate student researcher passionate about translational research in various fields. The following is an independent experimental study on genetic control of the cellular processes that regulate protein expression and transport in neurodegeneration. The study was conducted under the supervision of Dr. Derrick Gibbings in the Department of Cellular and Molecular Medicine at the University of Ottawa. Throughout the study, Philip conducted all experiments involved, in addition to collecting and processing the generated data.

The misfolding of the protein tau contributes to the development of Alzheimer's disease (AD). Misfolded tau is thought to propagate through a homeostatic degradation process known as autophagy, resulting in the export of cellular materials to the extracellular space via extracellular vesicles, called exosomes. By inhibiting the ATG7 and p62 genes necessary for autophagy to occur, the effects on the amount of exosomal and intracellular tau can be observed. Following the analysis of western blot and protein assay data, it was determined that the inhibition of the ATG7 and p62 genes results in a 70% and 60% reduction in the concentration of tau found in exosomes, respectively. These results suggest potential therapeutic applications of autophagic gene inhibition for the treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease which results in the disruption of communication between neurons, ultimately resulting in cerebral atrophy.[1] Upon observing the pathophysiology of the brains of patients afflicted with AD, one prominent feature is the dense aggregation of a misfolded version of the protein tau —which normally aids in the formation of microtubules —in large accumulations, known as neurofibrillary tangles, resulting in inhibited communication between neurons.[2] While tau in its misfolded form can be dangerous, it requires a pathway to be propagated through the extracellular space, away from the cell of origin to aggregate. Currently, one of the increasingly widespread hypotheses regarding the mechanism behind the propagation of tau and other prion-like proteins to the extracellular space is that they are carried as cargo in extracellular vesicles known as exosomes.[3]

Cells use a process called autophagy to maintain homeostasis in the presence of unwanted cellular constituents, including misfolded tau. During this process, cytosolic components and proteins are delivered to lysosomes for degradation. Occasionally, these cellular materials may also be loaded into exosomes to be released to the extracellular environment.[4]

Current treatments for AD are predominantly aimed towards alleviating symptoms by utilizing biological treatments such as cholinesterase inhibitors and behavioral approaches, which do not target the underlying mechanisms of neurodegeneration.[5] Thus, exploring the interactions between the autophagic pathway and exosomal export pathway involved in the propagation of misfolded tau is critical in both understanding the etiology of AD and in developing more effective therapeutics that target pathogenesis.

RESEARCH DESIGN

The objective of this study is to determine the effect of autophagic gene inhibition on the amount of tau in the exosomes of SH-SY5Y human dopaminergic neurons (SHSY cells). It is hypothesized that when cells are transfected with short interfering RNAs (siRNAs), which inhibit the expression of autophagic genes, the amount of tau found in the exosomes of SHSY cells will decrease significantly. siRNAs are double-stranded nucleic acids with short nucleotide sequences of around 21 base pairs. They are equipped with 3' overhangs that allow them to bind to mRNAs at specific sequences, promoting degradation of those sites, thus preventing the translation of specific proteins.[6]

The effect of autophagic genes on the amount of tau loaded into exosomes can be determined using siRNAs to knock down the expression of autophagy-related genes ATG7 and p62 in SHSY cells and observing the amount of tau that is loaded into the exosomes of SHSY cells compared to controls. If there is a significant difference in the levels of exosomal tau between the control and treatment groups, it could indicate that tau is loaded into exosomes through an autophagic pathway. Furthermore, this finding may indicate that by inhibiting autophagy in neurons, the spread of tau can be reduced significantly or stopped —a development that may be used to advance research regarding understanding and treating AD.

The ATG7 gene was chosen specifically because of its central role in autophagy, with the ATG7 protein acting as an enzyme that activates autophagic processes.[7] Thus, if its activity is downregulated, autophagic activity in a cell will be reduced. The p62 gene was selected due to its role in encoding the p62 autophagic marker, which has recently been shown to deliver ubiquitinated proteins,

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including tau, for degradation through autophagy.[8] Since both ATG7 and p62 are autophagy-related genes, any widespread effect can be attributed to autophagy-related genes as a whole, and any discrepancies can be attributed to the individual genes themselves.

CELL CULTURE

A fully confluent 10 cm plate of SHSY cells raised in Dulbecco's Modified Eagle Media was split into seven new plates: two control plates, two ATG7 knockout plates, two p62 knockout plates, and one plate for the passage of the cell line. Following incubation, each plate was transfected with its respective siRNA treatment (control, ATG7, and p62 siRNAs). Three days post-transfection, the media in each plate was replaced with culture lacking fetal bovine serum (FBS), after being washed with phosphate buffer saline (PBS) to remove the exosomes found in FBS. The plates were incubated for another day to allow for the release of sufficient amounts of exosomes. The media for each treatment group was collected to procure cell lysates and exosome samples. The supernatant from the collected media underwent serial centrifugation. After the complete removal of the supernatants, the exosome pellets for each sample were resuspended in 50 µL of PBS and stored at four degrees Celsius.

EXOSOME QUANTIFICATION

Using the ZetaView particle tracker machine and software, the particle sizes from the diluted pellets were determined and recorded in Table 1, confirming their identification as exosomes.[9]

PROTEIN QUANTIFICATION

The micro BCA protein quantification assay is a method used to normalize the amount of protein taken from each sample, so that any change in the amount of tau and other loading controls can be attributed to the siRNA treatment and not loading error. The cell lysates and exosome samples were serially diluted in cell lysis buffer and pipetted into a 96-well plate and in duplicate to ascertain protein assay results. The plate was placed in the Synergy H1 Hybrid Reader for analysis, where the signal given off by the bovine serum albumin (BSA) solutions of known concentrations were used as standards to compare with solutions of cell lysates and exosomes. This method allowed the concentrations of protein in the cell lysate and exosome solutions to be determined. Using this information, the corresponding amounts of PBS and lysis buffer for each cell lysate and exosome sample for each group was ascertained.

A western blot was used to visualize the amount of specific proteins in cells and exosomes. Proteins from the samples were separated by molecular weight through gel electrophoresis and subsequently transferred to a membrane. Antibodies for the protein in question were then bound to the membrane. A substrate was applied to the membrane that activates chemiluminescent markers on the antibodies, which were then imaged with the LG ImageQuant LAS, which captured the emitted chemiluminescent signals. Using the intensity of the signals within a given area, the amount of tau in each sample was quantified.[10] The membrane was washed again so that it was stripped of antibodies. It was then cut to test for the presence of the loading controls —TSG101 and flotillin-2 for exosomes, and tubulin and ALIX for cells. It was also tested for the autophagy marker LC3, and the protein SOD1 (implicated in ALS) using the same immunoblotting method.[10] Leftover cell lysates were tested for the ATG7 gene to confirm its knockdown.

TABLE 1. ZetaView exosome size, dilution, and particle concentration readings.

Trial 1	Particle Size (nm)	Concentration - particles/mL (reading)	Dilution Factor	Calculated Original Concentration
SHSY Control	187.6	2.60E+06	10000	2.60E+10
SHSY ATG7	233.7	2.70E+06	1000000	2.70E+12
SHSY P62	209.4	9.00E+06	10000	9.00E+10
Trial 2	Particle Size (nm)	Concentration (reading)	Dilution Factor	Calculated Original Concentration
SHSY Control	143.7	2.60E+06	1000	2.60E+09
SHSY ATG7	218.1	1.90E+06	250	4.75E+08
SHSY P62	215.6	2.00E+06	250	5.00E+08

TABLE 2. Percent reduction in exosomal tau for p62 and ATG7 gene knockdowns as compared to a control siRNA treatment group and standard deviation in loading controls.

TSG101 for SHSY Exosomes		
Intensity	Average	Intensity/Average
1790000	1733333	1.032692308
1660000		0.957692308
1750000		1.009615385

SHSY Exosome Tau Expression			
	Intensity	Intensity/Control	% Reduction
Control	84500		
ATG7	25800	0.305325444	~70%
p62	35400	0.418934911	~60%

RESULTS AND ANALYSIS

Using Fiji, the relative intensity of chemiluminescence from the blots was quantified. From the processed data in Table 2, it can be noted that the inhibition of ATG7 and p62 caused a significant decrease in the amount of exosomal tau (70% and 60% reductions, respectively).

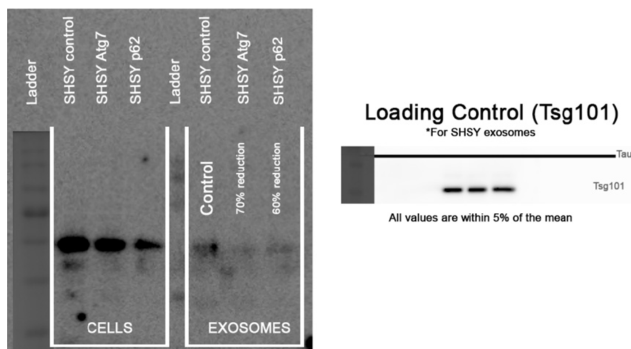

FIGURE 1. Results for western blot for tau with loading control. The western blot depicts bands of varying intensity representing varying concentrations of tau within the cells and exosomes of the control and knockout groups. Loading control confirms elimination of attribution of results to loading error.

Figure 1 demonstrates visible reduction in exosomal tau following the inhibition of ATG7 and p62 in SHSY cells, confirming the hypothesized reduction in the loading of tau into exosomes through autophagic processes.

SHSY Exosomes			
flotillin-2	ALIX	SOD1	LC3
SHSY Cells			
tubulin		LC3	

TABLE 3. Additional western blots for other proteins and loading controls.

With two other loading controls in Table 3 confirming the results for exosomal tau, the trends exhibited in the exosomes are quite reliable. Contrarily, the loading controls show uneven loading of cellular protein; therefore, no conclusive trend can be drawn from the data for protein expression in SHSY cells. In the exosomes, SOD1 showed no significant difference following Fiji analysis, providing evidence that this phenomenon is not applicable towards all prion-like proteins and that SOD1 may not be loaded into exosomes via the autophagic pathway. LC3 is an autophagic marker that has two forms with different molecular weights; depending on the activity of autophagy, one form can be downregulated while the other is upregulated, but this cannot be discerned with the blot performed as the band for LC3 had not yet separated.[11] In future trials, a lower-percentage resolving gel for faster separation of protein bands should be used to allow the two bands of LC3 to be distinguished, permitting the amount of autophagy occurring to be more directly measured. Multiple controls were used to reduce random error, including a control siRNA, blotting for loading controls, and quantifying and standardizing protein concentrations using a protein assay.

A western blot was also performed to test for the presence of ATG7 in the leftover cell lysates of the control group and the cells that underwent the ATG7 siRNA treatment, confirming the knockdown of this autophagic gene.

DISCUSSION

The results of this study prompt further research into the relationship between the autophagy pathway and the spread of tau and other prion-like proteins; through the relatively unchanged expression of SOD1 in the exosomes of both the control and treatment groups, it is demonstrated that not all prion-like proteins responsible for other neurodegenerative diseases are spread via an autophagic pathway. Hence, the results of this study may only reflect a unique downregulation in the loading of tau into exosomes, possibly affecting the progression of AD. There are, however, factors other than misfolded tau that contribute to the onset and progression of AD.

Although p62 and ATG7 are crucial autophagic genes, the assumption that their effects are representative of all autophagic genes was made. Moreover, only one complete trial was performed. More replicates of the experiment are required to ascertain the trends exhibited.

Despite the visible reduction in exosomal tau following the inhibition of ATG7 and p62 in SHSY cells, the inhibition of autophagy can lead to increased levels of apoptosis, which may be damaging in other ways.[12] Hence, while therapeutic applications of these results are still distant objectives, the significance of the interplay between autophagic genes and the loading of tau into exosomes lends itself to further research.

CONCLUSION

The inhibition of the autophagic genes ATG7 and p62 in SHSY cells caused a 70% and 60% reduction in the amount of tau that is loaded into exosomes, respectively. If applied to the development of therapeutic treatments, this procedure can inhibit the spread of misfolded tau and prevent the formation of neurofibrillary tangles —potentially slowing down or even halting the progression of AD.

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Atreyee De is a pre-doctoral student at Dr. Bhagwati Gupta's lab in McMaster University. She is currently studying the insulin signaling and Wnt signaling pathways of model organisms nematode Caenorhabditis elegans and Caenorhabditis briggsae to understand the role of these pathways in the development, metabolism, and longevity of the worms. She will begin her PhD in fall 2022.

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