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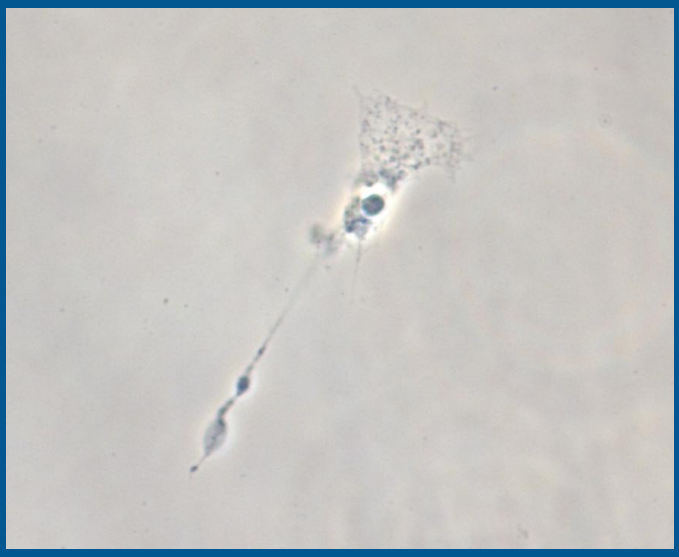

Assessing the changes in autophagic activity in living sympathetic neurons treated with beta amyloid

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Introduction:

Autophagy is a major cellular degradation pathway by which the cell renews and clears itself from damaged organelles and aggregated proteins (Walker, 2000). When abnormal or misfolded proteins aggregate, they tend to cause synaptic dysfunction and damage to the organelles (Walker, 2000). Post-mitotic neuronal cell survival depends highly on autophagy as misfolded proteins and damaged organelles cannot be diluted through cell division (Tooze, 2008). An autophagy pathway that is defective has been observed in neurodegenerative diseases and in various types of cancer (Mizushima, 2008). Thus the up-regulation of autophagy has been studied as a potential treatment for neurodegenerative diseases (Sarkar, S., 2013).

One of the major steps in the process of autophagy is the fusion of autophagosome with the lysosome followed by proteolytic degradation of the autophagosome contents (Chikte et al, 2014). Lysosomal proteases export products of degradation back to the cytoplasm (Sarkar, S., 2013). In multiple studies of autophagy, lysotracker, a fluorescence vital stain, was used to label acidic spherical granules within cells. These granules are not only lysosome specific, but contain autophagosomes as well. Therefore, this method is used to measure autophagic activity and analyze the effects of different treatments on autophagy (Chikte et al, 2014). In addition, the early onset of Alzheimer's disease (AD) has been linked to mutations in Beta amyloid (Aβ) A4 protein precursor (APP) which form aggregates that grow continuously and disturb cell processes including autophagy (Maltsev et al, 2011).

Autophagy is directly related to the increased expression of Aβ protein and to the dysfunction of Aβ clearance in AD (Ferrer, 2001). Aβ plaques are one of the major products present in the brains of patients with AD. There are a few mechanisms by which these accumulate. The early onset forms of AD are associated with mutations in APP. Mutations can also happen in presenilin-1 (PS1) or presenilin-2 (PS2) which can be the catalytic subunits of γ-secretase, which is one of the final components within the pathway that results in generating Aβ (Murphy, 2010). AD is characterized by increased amounts of soluble and insoluble Aβ. These high levels of undegraded Aβ can initiate a series of events that culminate in neuronal damage or death resulting in the progressive clinical dementia of Alzheimer's (Mawuenyega, 2010).

Therefore, in this study I performed experiments that included Aβ 25-35 fragment and Aβ 25-35 fragment treatment. Both fragments are 10 amino acid long proteins, 25-35 being the active one found in humans and 35-25 being the inactive one used as a control in this experiment. The treatments were examined in terms of their effects on the autophagic process by measuring levels of lysosomal activity with the lysotracker® Green DND-26 labelling.

Autophagic activity in neurons was studied in the embryonic peripheral neurons of the *Gallus gallus* embryo. My hypothesis in this study is that autophagy will be decreased in neuronal cells by adding extracellular Beta amyloid as measured by lysosomal activity.

Materials and Methods:

Cover Slip Sterilization

The protocol to sterilize and clean cover slips for this experiment was performed as described by Morris (2015a).

Dissections

Dissections of dorsal root ganglia (DRGs) and sympathetic nerve chains were performed as described by Morris (2015a). Neurons were collected from chick embryos to be used as primary cultures for this study. Embryos used were 10 day old *Gallus gallus.*

Observing live cells using chip chambers

The chip chambers with live cells were made and observed as described by Morris (2015b).

Beta Amyloid Solution Preparation and Treatment

Two Aβ fragments were employed in the current study: the active 25-35 for the experimental condition and the inactive 35-25 for the control condition. Both were ordered from Sigma-Aldrich and have arrived as powder. The stock solution of Aβ 25-35 was made by adding 377 µL of DMSO into the tube with Aβ, reaching the final concentration of 25 µM. The stock solution of Aβ 35-25 was made by adding 94 µL of DMSO into the tube with Aβ, reaching the final concentration of 25 µM. The working solutions for treatment for both fragments were made by adding 20 µL of the stock solution into 1 ml of growth medium (a 1:50 dilution) already added into an EP tube. The treatment concentration accomplished by this method was 25 µM for each condition. Working solutions were made immediately before the treatment in order to avoid the aggregation of Aβ in the growth medium.

Cells were plated in petri dishes in growth medium and left to grow for 24 hours in the incubator. 1 ml of growth medium was removed from 2mls to leave behind 1ml on the cells. Then 1 ml of Aβ working solution was added to the dish. Petri dishes were then put back into the incubator for 12 hours after which they were labeled with lysotracker.

Lysotracker Labeling

A working solution for lysotracker® Green DND-26 was utilized to measure lysosome activity in the Aβ treated cells. 1 µl of 1 mM stock solution was diluted into 17 ml HBSS in order to get the 60 nM working lysotracker solution. After Aβ incubation, all of the growth medium was removed from the petri dishes. Cells were washed with HBSS warmed to 37.5°C, for one minute. After HBSS was removed, 1 mL of lysotracker solution was placed in each petri dish. They were placed in the incubator and kept out of light for 5 minutes. After incubation, all of the lysotracker-containing medium was removed. Cells were washed three times with warm HBSS, for 1 minute per each wash. Finally, 1 mL of fresh growth medium was added to the petri dishes.

Data Collection and Analysis

SPOT Imaging Software connected with InSight Firewire 2 Megasample camera, on a Macintosh computer, was used to obtain the images. Both transmitted and fluorescent images were captured using this program. Cells were exposed to fluorescence for approximately 20 seconds. Images of neurons were taken using a Nikon Eclipse E200 at 40x magnification. Next, these images were quantified using Image J32 program. Due to variations across each slide, thresholds and exposure times were maintained constant across each slide. First, cell boundary of a single neuron in the transmitted image was circumscribed with the freehand trace tool and transferred to the corresponding fluorescence neuron image. In order to adjust the threshold of the image, the intensity command within the Image tab was set between 32 and 255. The image type was set to 8-bit grey. Within the Analyze tab, particle size was set to 1-infinity and the following options were checked - display results, clear results, summarize, in situ show. This allowed for the retrieval of the % area value of measured area (red pixels) in the fluorescent image. The % area of pixels represents lysosomes that have a greater threshold than the overall neuron surface that was selected before. This data was read from the % area column in the displayed results table and the raw data was then averaged to determine the condition with the highest level of lysosomal activity (This quantification was achieved in collaboration with Keran Yang).

Results:

This study found that the Aβ 25-35 treatment decreased autophagy as measured by lysosomal activity. The % area of pixels of the lysosomes measured in relation to the overall selected area of the neuron was obtained for 7 neuron images in each condition. The averages of these results were calculated and presented in Figure 3. The average % area for Aβ 35-25 treated neurons is 32.633 compared to 24.622 average % area for Aβ 25-35 treated neurons. Figure 1 and Figure 2 represent a neuron selected between 7 images in each condition. As seen in Figure 1, the fluorescent green light, indicating lysosomal activity in the neuron, appears brighter compared Figure 2.

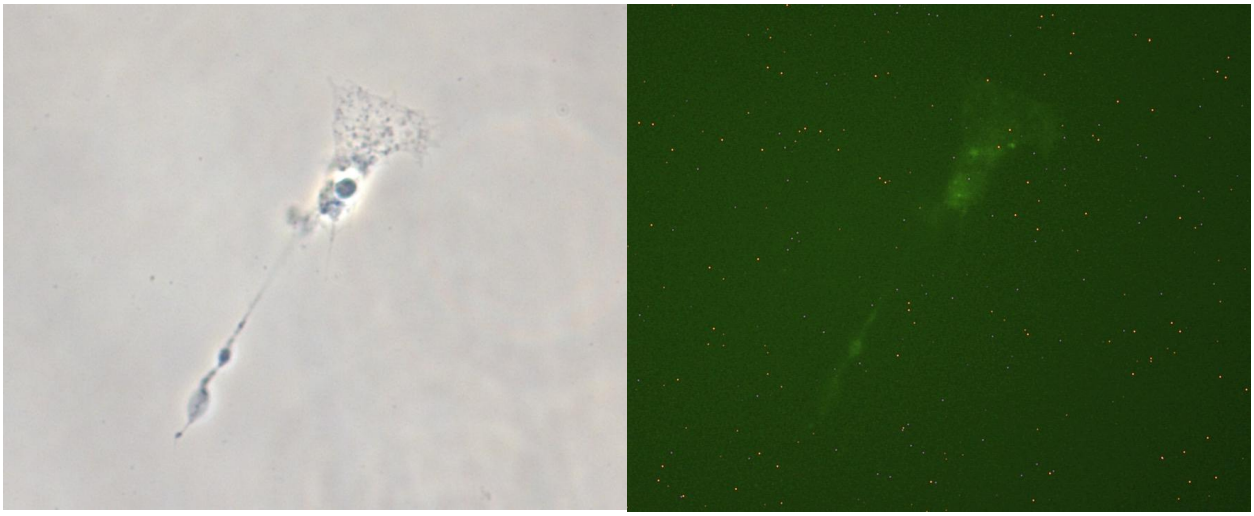

Figure 1. Lysosomal activity in Aβ 35-25 treated neuron. This cell was treated with 25μM concentration of Aβ inactive fragment solution. The image on the left side is the transmitted image and the one on the right is the fluorescent image. The fluorescent light represents the lysosomal activity in the neuron soma and axon, as indicated by bright green acidic spherical granules. Cell body appears brighter than the axon.

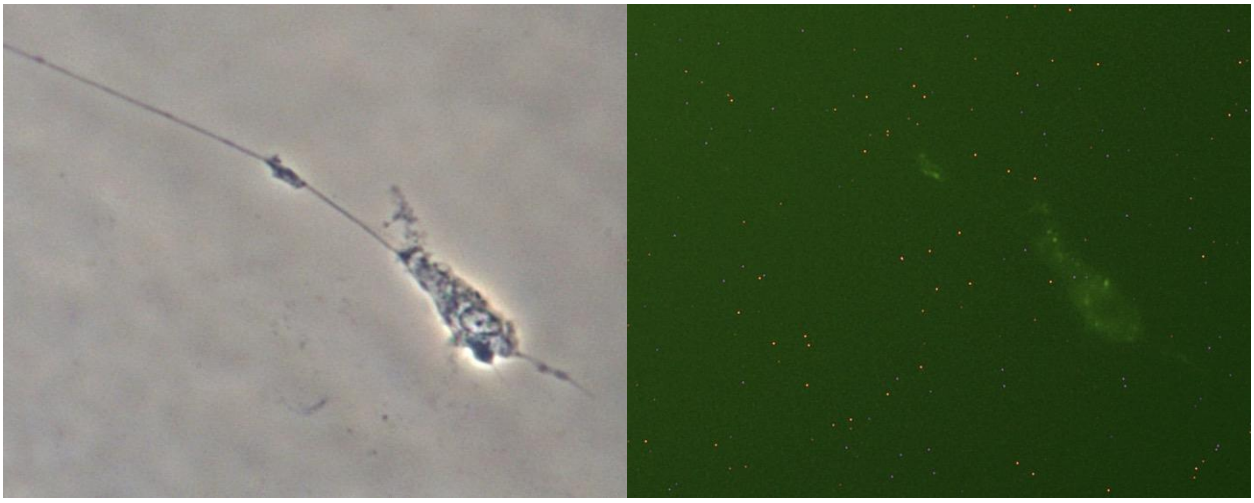

Figure 2. Lysosomal activity in Aβ 25-35 treated neuron. This cell was treated with 25μM concentration of Aβ active fragment solution. The image on the left side is the transmitted image and the one on the right is the fluorescent image. The fluorescent light represents the lysosomal activity as indicated by bright green acidic spherical granules in the cell body. Notice the decrease in brightness in the cell body compared to the control image (Figure 1). The brightest fluorescent light corresponds to highest lysosomal activity.

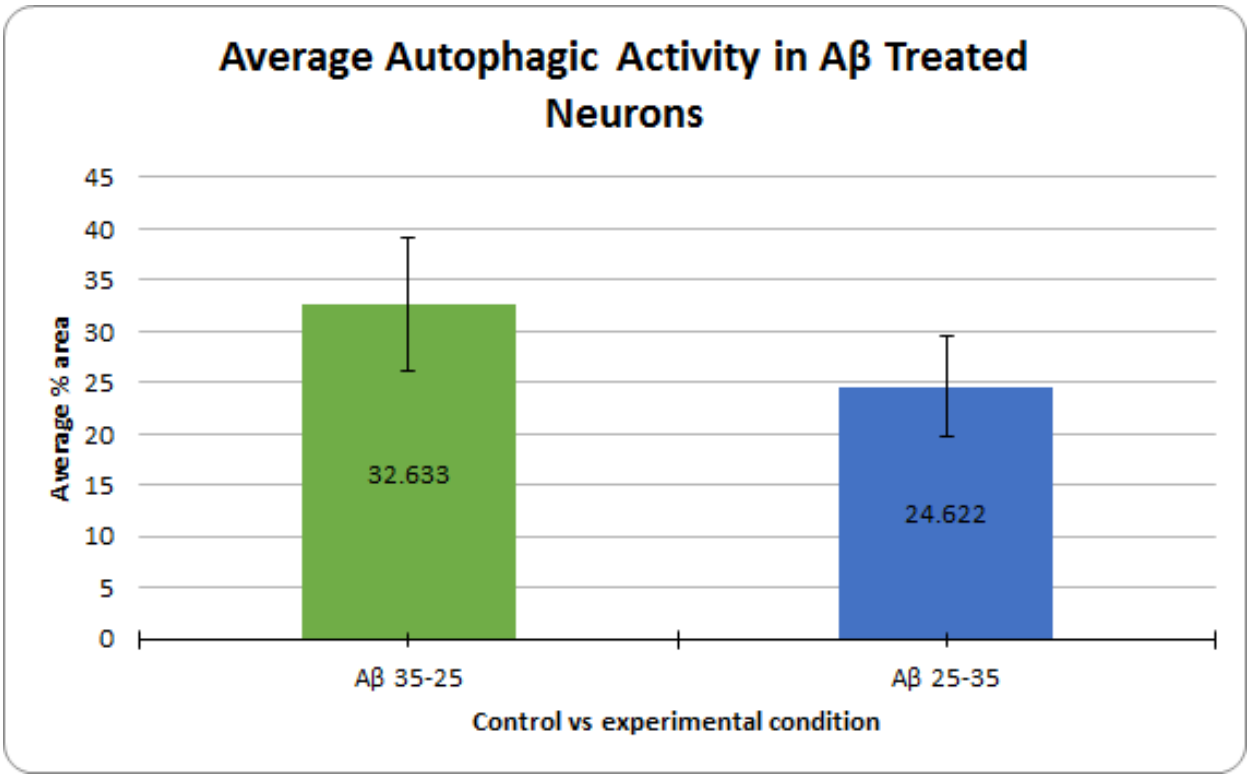

Figure 3. Autophagy in Aβ treated neurons as measured by lysosomal activity. The y-axis is the average % area of the pixels of the lysosomes: it represents the autophagic activity in neurons. Data for the Aβ 35-25 bar were averaged from measurements of 7 cells on 2 coverslips from 1 trial and data for the Aβ 25-35 bar were averaged from measurements of 7 cells on 2 coverslips from 1 trial. The experimental bar is reduced by 24.5 % compared to the control bar.

Discussion:

In the current study, autophagic activity appears to be reduced in neurons with the Aβ 25-35 experimental treatment as compared to Aβ 35-25 control treatment. This supports my hypothesis that autophagy will be decreased in neuronal cells by adding extracellular Beta amyloid as measured by lysosomal activity.

As seen in Figure 3, the % area of pixels of the lysosomes measured in relation to the overall selected area of the neuron was lower for cells treated with Aβ 25-35 than the % area of pixels of the lysosomes of the cells treated with Aβ 35-25. This data generated from a sample of 4 petri dishes suggests that Aβ disrupts the normal functioning of the autophagic process and the lysosomal activity in neuronal cells.

If the sample collected was larger and the data generated supported the current trends seen in this experiment, the study of the effect of Aβ on AD and other neurodegenerative diseases could be developed further. New findings would provide additional data to examine the hypothesis that Beta amyloid negatively affects the process of autophagy which is crucial in order to maintain the homeostasis of the cell (Walker, 2000). When the autophagic activity is

reduced, damaged organelles and aggregated proteins, one of which is the Aβ protein that accumulates in neurons of patients with AD, cannot be degraded to bring the cell back to its normal functioning (Mizushima, 2008). Therefore, if the larger sample supported current findings this would corroborate the model that the neuronal homeostasis was disrupted by the introduction of Aβ 25-35. Subsequent analysis of possible treatments that would decrease or completely erase these undesired effects should be examined. One of these treatments could be using the newly screened and studied AUTEN-99, small autophagy enhancer molecule, that showed to induce autophagic activity and repress oxidative stress effects induced by adding H2O2 (Kovacs, 2017). However, it is pivotal to test cells' responses by looking at how it affects processes and compounds more specific to neurodegeneration to be able to validate it as a potential treatment.

Even though experiments have been done using Aβ 25-35 fragments in relation to autophagy in other culture systems (Yang, 2014), one of the improvements for the next time this experiment is performed is using Aβ 1-42 because Alzheimer's disease (AD) is characterized by increased amounts of Aβ, predominantly in the form of Aβ 1-42 in amyloid plaques and Aβ 1-40 in amyloid angiopathy (Mawuenyega, 2010). These are the two main forms of the peptide that are normally produced during the APP processing, hence this change would be beneficial as these peptides are actually present in senile plaques and thus provide more physiological relevance to changes in patients' brains (Mawuenyega, 2010).

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I have abided by Wheaton College Honor Code in this work.

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