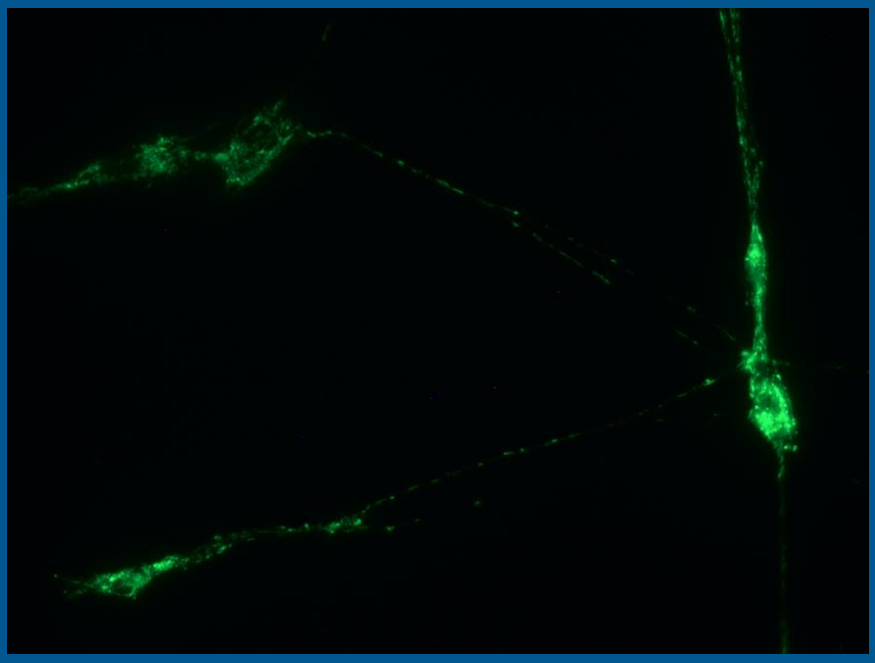
Wheaton Journal of
Neurobiology Research

Issue 10, Fall 2017:

"Modeling disease using primary neuronal tissue culture"

R.L. Morris, Editor. Wheaton College, Norton, Massachusetts.



Preliminary study of the effects of exogenous amyloid-beta protein 25-35 on mitochondrial function in glial cells of *Gallus gallus*

Ryan J. McKeon

BIO 324 / Neurobiology
Final Research Paper
4 December 2017

Preliminary study of the effects of exogenous amyloid-beta protein 25-35 on mitochondrial function in glial cells of *Gallus gallus*

Ryan J. McKeon
Final Research Paper written for
Wheaton Journal of Neurobiology Research
BIO 324 / Neurobiology
Wheaton College, Norton Massachusetts
4 December 2017

Introduction:

Alzheimer's disease (AD) is the most common neurodegenerative disease in the increasing population of elderly people and geriatrics. The causes of the disease are still widely unknown, but current studies are focusing on the effects of specific proteins, such as amyloid-beta (AB), on cellular organelles and mechanisms. The question of whether beta amyloid protein impacts the functionality of mitochondria in neuronal and glial cells is important in relation to the study of Alzheimer's. A well-known characteristic prevalent in AD patients is the accumulation of plaques in the brain (Murphy, 2010). These plaques are predominantly consisted of aggregates of amyloid-beta, which is a peptide produced by proteolytic cleavage of the amyloid precursor protein (Finder & Glockshuber, 2007). It has been suggested that amyloid plaques appear early during AD but their origin, mode of formation, and contribution to Alzheimer's pathogenesis is still controversial (Nagele et al., 2004). A study done by Carla Shatz at Stanford University suggests that beta-amyloid aggregations are small, soluble clusters that can travel freely in the brain. The study implied that AB in this clustered form may bind to receptors on nerve cells, initiating degradation and the eroding of synapses between nerve cells, making them unable to communicate and transmit electrochemical impulses (Goldman, 2013).

Mitochondria play a key role in preventing neurodegeneration by being responsible for regulating energy metabolism and cell death pathways. The primary function of a mitochondrion is oxidative phosphorylation to make cellular energy in the form of ATP (Modica-Napolitano & Aprille, 2001). Mitochondria are necessary to establish membrane excitability, initiate neurotransmission, and establish neuroplasticity in the brain (Kann & Kovács, 2007). Many studies suggest that mitochondrial dysfunction plays a significant role in excessive oxidative damage, which may lead to the onset of AD (Moreira, 2010). This study conducted by Moreira provides evidence that mitochondria are contributors to AD because, when not functioning properly in neurons and glia, the cell cannot properly respire to maintain cell health or undergo the organized process of apoptosis, programmed cell death. In addition to this, a link was discovered between mitochondrial dysfunction and autophagy, internal consumption of the body's tissues. Improperly functioning mitochondria may produce oxidatively-damaged macromolecules and organelles that may trigger the accumulation of lipofuscin that can promote neuronal dysfunction and degeneration (Kann, 2007). Lipofuscin is an electron-dense

fluorescent material known to accumulate in the lysosomes within neurons (Terman & Brunk, 1998).

Mitochondrial function will be examined by introducing the cells to amyloid-beta and staining them with Rhodamine 123 (Rh-123) to visualize the effects. Rh-123 is a lipophilic cation that penetrates the mitochondrial membrane and acts to fluorescently-label the mitochondrion. Modica-Napolitano et al. (2001) states that lipophilic cations are "concentrated by cells and into mitochondria in response to negative inside transmembrane potentials" (Modica-Napolitano & Aprille, 2001). The Rh-123 is electrically attracted to the negative charge within the cell membrane, allowing it to get into the cell. It will then be attracted to the negative charge within the mitochondrial membrane. This is the mechanism by which Rh-123 is translocated to reside within the mitochondria, making individual mitochondria visible when observed using fluorescent microscopy (Baracca, Sgarbi, Solaini, & Lenaz, 2003). If this process cannot occur, then this may imply that the mitochondria are not functioning properly and, therefore, cannot be fluorescently-labeled. The cells will not be effectively stained with Rh-123 if they are not attracted to the inner membrane of the cell and of the mitochondria (Modica-Napolitano & Aprille, 2001). This is expected to be seen in the cells treated with amyloid-beta 25-35. AB 35-25 is the reverse version of AB 25-35 and will be used as the control because it is not activated.

Glial cells were chosen because research has shown a relationship between glia and beta-amyloid degradation which helps fight AD (Black, 2017). Research suggests that microglia activation may be linked to amyloid-beta plaque development. Nagele et al. (2004) claims that microglia are believed to "rapidly migrate into and congregate with" plaques, but fail to diffuse plaques (Nagele et al., 2004). This research suggests that microglia appear to contribute to the morphological evolution and development of the amyloid plaques that may be linked to AD. Based on these research results, it is hypothesized that the presence of exogenous amyloid-beta protein (fragment 25-35) negatively influences the mitochondrial function in four-day and nine-day glial cells.

Materials and Methods:

Primary culture dissection of chick embryos. The dissection of the chick embryos to get dorsal root ganglion was performed following the protocol devised by Robert L. Morris based on the protocol by Peter J. Hollenbeck. This was presented in "Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION" (Morris, 2017). This procedure was performed on ten day old chick embryos. Cultured cells were plated sparsely to clearly observe individual cells. Nine-day cells were treated in trial one and four-day cells were treated in trial two. This was done due to cell availability in cooperation with collaborators.

Preparation of growth medium. Growth medium was prepared by Robert L. Morris. Neurons and glia were grown in an adapted form of Leibovitz L-15 that created an F-plus medium containing nerve growth factors (NGFs). Penicillin/streptomycin (pen/strep) was used to avoid any bacterial contamination (Morris, 2017).

Introduction of exogenous AB to cultured cells. AB fragments 25-35 (for experimental) and 35-25 (for control) were added to nine day cells (trial one) and four day cells (trial two). For the forward version, a 25μM working solution was created using a 2.5μM stock solution made by adding 1mg of solid AB to 377μl of DMSO. The working solution was made by adding 10μl of the 2.5μM stock solution to 1ml of growth medium. This is a 1:100 dilution that created 1ml of 25μM AB 25-35. For the reverse version, the stock solution was made by adding 250mg solid AB to 94nl of DMSO to create the 2.5μM stock. The working solution was created the same way from this stock solution as in the 25-35 (Allaman et al., 2010). For the control in trial two, 10μl DMSO was added to 1ml of growth medium to account for an issue with the AB 35-25 that arose. Methods done in collaboration with Omar Raouf.

Incubation of treated cells. The cells treated with both AB 25-35 and Ab 35-25 were incubated for 24 hours at 37 degrees C. Methods done in collaboration with Omar Raouf.

Staining of cells using Rh-123. The working solution of Rh-123 was made adding 1μl of 1mg/ml Rh-123 to 1ml of growth medium in a 1:1000 dilution to create 1ml of 1μg/ml working solution Rh-123. The cultured cells were removed from the incubator and the growth medium containing the cultured cells was removed by a full exchange to add the 1ml Rh-123 working solution. Cells with applied Rh-123 were then incubated for ten minutes at 37 degrees C. After incubation, cells were then washed four times with HBSS (Black, 2017) (Humure, 2017). Methods done in collaboration with Omar Raouf, Daniel Southerland, Stephanie Martin, Matt Morgan, and Luis Lazo.

Making chip chambers. This was based on protocol by Robert L. Morris. Microscope slides were placed on a paper towel and coverslip chips were placed in the center of the slide in the shape of a coverslip. A drop of growth medium was placed in the center. The coverslip with treated cells was removed from the plate, wiped with a KimWipe to remove excess HBSS, and placed cell-side down onto the chips. Any bubbles or excess growth medium was removed, and the edges of the coverslip were painted with valap sealant to seal the edges (Morris, 2017). Methods done in collaboration with Omar Raouf.

Fluorescent imaging of cells. After making the chip chambers, cells were observed under the microscope to obtain transmitted and fluorescent images in the ICUC at Wheaton College in Norton, Massachusetts. A Nikon Eclipse E400 microscope with an Insight Firewire camera mount positioned on top of the microscope was used to analyze the cells. The images were taken using SPOT software (version 5.2) on the "Taurus" computer. All images were taken at 40x magnification at phase two and only two images were taken per cell or grouping of cells to avoid bleaching. All images were taken on the same day the Rh-123 was added and incubated. All cells imaged were the same age on the day they were imaged for each trial (i.e. all cells imaged in trial one were nine-day cells and all cells imaged in trial two were four-day cells). Exposure times were set at 1050ms for the AB 35-25-treated group but had to be adjusted for the AB 25-35 group that did not fluorescently illuminate at this exposure time. Mitochondria in the 25-35 group could not be located under the 1050ms exposure, so all exposure times for this group were set at 1575ms so that mitochondria could be quantitatively-analyzed. A total of 14 images were taken through the two trials. Methods done in collaboration with Omar Raouf.

Data analysis and quantification. Images were quantified using ImageJ software, version 10.2. The quantified region of interest was determined from cell shape and appearance in transmitted light images. This same region was then circumscribed in the fluorescent image using the free selection segmented-line tool to quantify brightness of mitochondria within the region. The brightness of the background staining in a similar area outside of the cell was determined by moving the circumscribed boundary to a region beyond the edge of the cell that was still within one cell diameter. Data was analyzed and measured by the software to produce a mean brightness for the enclosed area selected. The difference between the two numbers was taken and this number represented the brightness intensity (in arbitrary units) of the mitochondria with noise subtracted from signal. This was done on each image to quantify the brightness of the mitochondria. The ratio between the signal and noise of the 25-35 and 35-25 groups were used to properly quantify and avoid bias due to the different exposure times that were needed to observe the 25-35 group.

Results:

Observations were made by examining a total of 14 images combined from the two trials of the experiment conducted. Trial one was conducted using nine-day cells and trial two was conducted using four-day cells. A control (AB 35-25) and experimental (AB 25-35) image from each cell age are presented below (Figure 1, 2, 3, and 4). The images show a clear disparity between the brightness of the cells treated exogenously with AB 35-25 (DMSO for four-day cells) and AB 25-35 in both the nine-day and four-day cells. 35-25 groups have a much higher brightness intensity than the 25-35 groups which appear qualitatively dimmer. This can be seen in any of the images shown below. Figures 1 and 2 are the results from the nine-day cells, so the fluorescent images in these two figures can be compared to see the difference. Figures 3 and 4 were the images taken of the four-day cells. Fluorescent images for the 35-25 treated cells were taken with an exposure time of 1050ms (Figures 1 and 3). The fluorescent images for the 25-35 treated cells were unable to be examined at this exposure time, so exposure for these images was bumped to 1575ms to be quantitatively analyzed (Figures 2 and 4). Without changing the exposure time, mitochondria could not be located at the 1050ms exposure for the 25-35 treated cells. Examining the ratio between axonal brightness in both conditions limited bias caused by the difference in exposure used. This was taken into account when calculating mean brightness and comparing the conditions for both nine-day and four-day cells. All of the fluorescent images used in this study were the second image taken to avoid any further bleaching that may skew the results. The second image was chosen as the baseline for this study because focus needed to be adjusted fairly often after the first image was taken, and this created clear images that would be easily analyzed. These data were gathered in collaboration with Omar Raouf.

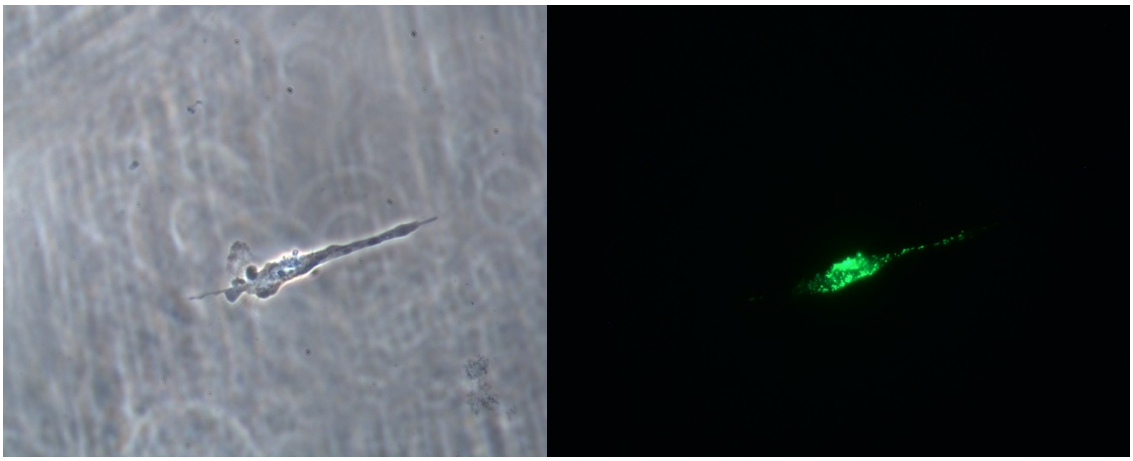


Figure 1: Transmitted and fluorescent images for the AB 35-25 group from trial one (nine-day cells). The same glial cell is represented in both images. From the fluorescent image with an exposure time of 1050ms, it is evident that the Rh-123 was able to pass through the glial membrane and the mitochondrial membranes. This may indicate functional mitochondrial activity as a result of being treated with AB 35-25. Two measurements were taken for the control condition of the nine-day cells (n=2). Images taken in collaboration with Omar Raouf.

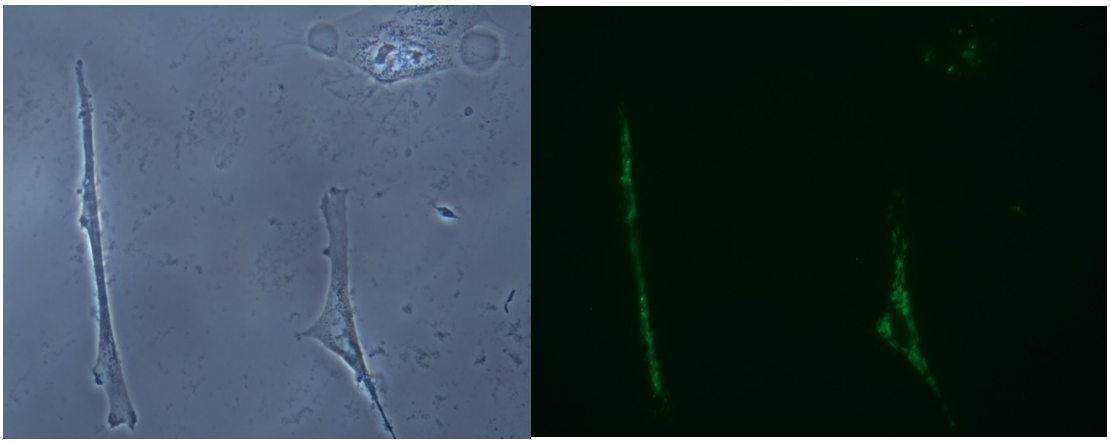


Figure 2: Transmitted and fluorescent images for the AB 25-35 group from trial one (nine-day cells). Multiple glia are visible and the fluorescent image with an exposure time of 1575ms shows a clear disparity between this image and the control above. The much dimmer mitochondria in this fluorescent image may provide evidence that the mitochondria are less functional and are not able to efficiently take up the Rh-123. These cells were treated with AB 25-35. Notice the aggregates of AB littered throughout the transmitted image. Three measurements were taken for the experimental condition of the nine-day cells (n= 3). Images taken in collaboration with Omar Raouf.

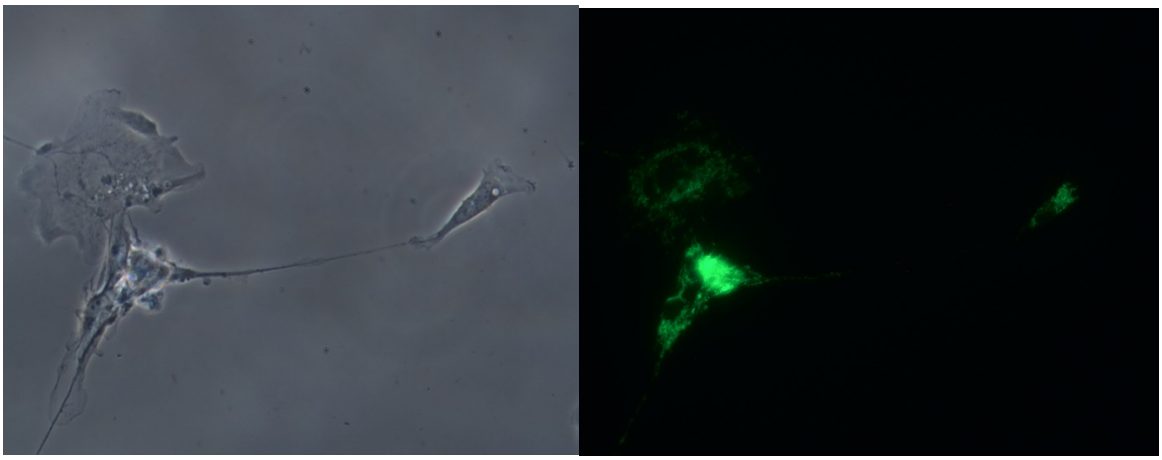


Figure 3: Transmitted and fluorescent images for the control group from trial two (four-day cells). These cells were treated with DMSO as a result of a complication with the AB 35-25. The glia here appear to be stained very well with the Rh-123, indicating that they have likely maintained their functionality (exposure time was 1050ms). Notice the formation of axons connecting the cells that were not seen in the images taken of nine-day cells. Six measurements were taken for the control condition of the four-day cells (n=6). Images taken in collaboration with Omar Raouf.

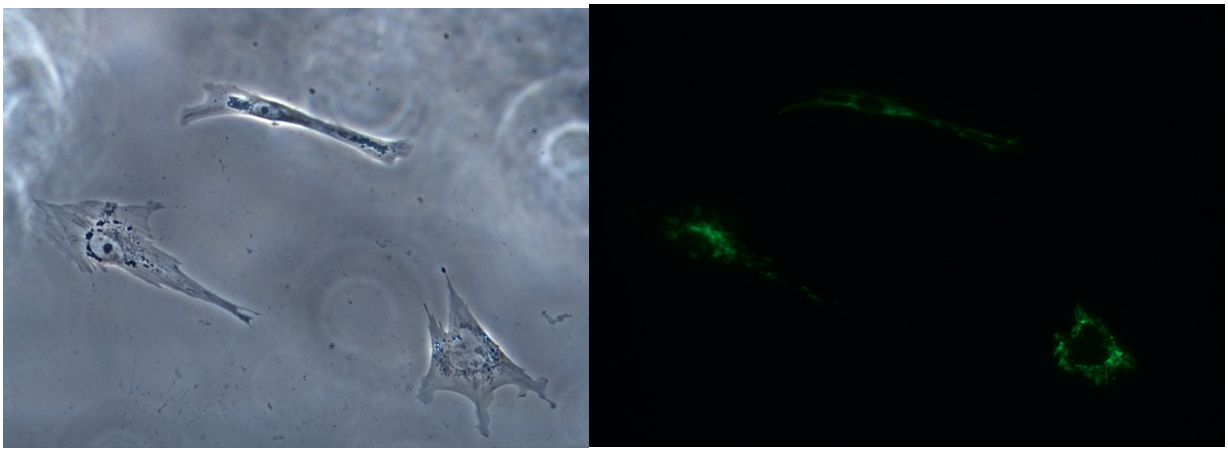


Figure 4: Transmitted and fluorescent images for the AB 25-35 group from trial two (four-day cells). Three glia are present here, and all appear noticeably dimmer than in the control group for this trial. Notice how little of the actual shape of the cell can be made out from the fluorescent image (exposure time was 1575ms). This may be evidence in favor that the mitochondria did not properly take up enough Rh-123 due to their inactivity from being treated with AB 25-35. Nine measurements were taken for the experimental condition of the four-day cells (n=9). Images taken in collaboration with Omar Raouf.

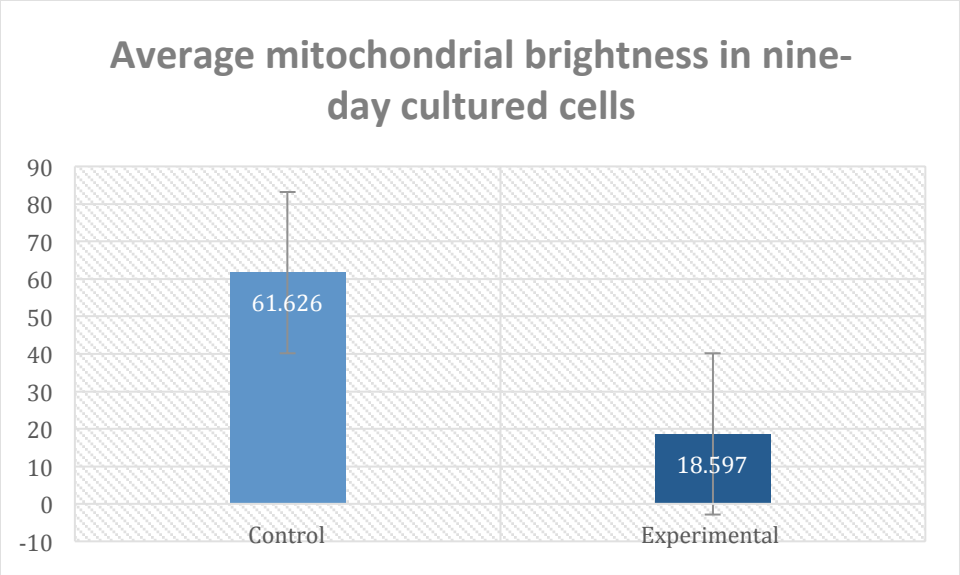


Figure 5: Graph showing the average mean brightness of the nine-day glial mitochondria that were analyzed. ImageJ software was used to gather the brightness levels of two control conditions (treated with AB 35-25) and three experimental conditions (treated with AB 25-35). It is clear that the control group was substantially brighter than the experimental cells treated with AB 25-35.

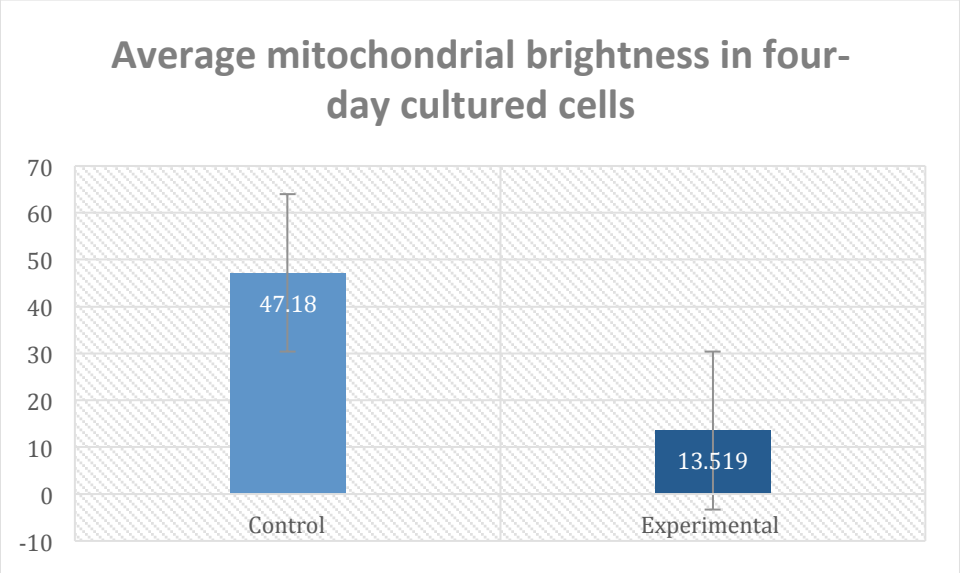


Figure 6: Graph showing the average mitochondrial brightness of glia measured from four-day cells. Six control conditions (treated with DMSO) and nine experimental conditions (treated with AB 25-35) were analyzed to gather these data. Again it is clear that the control group had a higher brightness intensity than the experimental group. A difference arises in that these numbers are lower than in nine-day. This could be a result of cellular damage to the four-day cells used in this trial as opposed to the nine-day cells used in trial one.

Discussion:

The data collected in this preliminary experiment show evidence in support of the hypothesis that the presence of exogenous AB 25-35 negatively affects mitochondrial function in glial cells. Although the data cannot be found to be statistically significant due to the wide deviation range and small sample size, the hypothesis can be supported based on the trends observed in the two trials. The test for mitochondrial function was done by observing the mean brightness intensity of mitochondria in glia following the Rh-123 staining. If the mitochondria were functioning properly, then the Rh-123 cation would have been more attracted to the negative charge within the mitochondria, allowing the Rh-123 to illuminate the individual mitochondrion. All images show that this occurred but at different intensities, with the illumination of the AB 25-35 groups being visibly less intense. The need to change exposure times for the 25-35 groups may also provide evidence in favor of the hypothesis. That the individual mitochondria in this group could not be observed without increasing exposure may help support the claim that the presence of active AB negatively affects mitochondrial function. This may be indicative that the mitochondria within the glial cells of the AB 25-35 treated group were more dysfunctional than those in the 35-25 groups. This analysis is applicable for both the nine-day and four-day glial cells examined.

If this experiment was repeated to reveal the same results every time, then this could be significant evidence that amyloid-beta protein is a key contributor to mitochondrial dysfunction, specifically the loss of function in central nervous system cells such as neurons and glia. This may be a result of limited ATP production within cells, which makes it difficult for the body to carry out vital body functions. From a cellular standpoint, having a real trend between AB 25-35 treated cells and AB 35-25 treated cells may imply that the exogenous application of AB 25-35 to cells could possibly induce the formation of plaques that may cause mitochondrial function to decrease (Humure, 2017). AB plaques present in the cell may lead to the disruption of functioning mitochondria that could cause a decreased production of ATP. Shevtzova et al. (2001) found similar results that AB may negatively affect mitochondria. This study found that AB causes the activation of specific channels that, when opened, culminate in the apoptotic death of cells. This may help contribute to neurodegeneration, the major characteristic of Alzheimer's disease (Shevtzova, Kireeva, & Bachurin, 2001). If this is true, then my results (with statistical significance) could likely be compared to those gathered in this study to provide more evidence in favor of the claim that mitochondrial dysfunction may be a contributor to neurodegeneration.

Rh-123 is light-sensitive, so a concerted effort was made to avoid any unnecessary exposure to light. An error could have come when introducing the cells to the Rh-123. Although a point was made to keep the cells with Rh-123 covered from light at all times, there is always the possibility that some light got to it that should not have. Another error may have occurred when doing the full exchanges of growth medium. Cell death was initiated the moment liquid was fully removed and it is possible, though unlikely, that some cells were negatively affected during this short period.

Future experiments could look at how different concentrations of AB 25-35 influence the functionality of the cell. Since only one concentration of 25μM AB was used, it could be helpful to examine how higher or lower concentrations impact mitochondria. Doing so could provide more evidence that AB is responsible for neurodegeneration. If higher concentrations of AB are found to produce more negative effects on mitochondria than

lower concentrations of AB, this could provide more evidence about the significance of AB and AB plaque formation on neurodegeneration. To refine this preliminary experimental design, I would use a larger sample size and try to use a different fragment of AB and see if that has any effects on function like the 25-35 fragment did. The larger sample size will help provide more significance to the results gathered. It would also help significance to take measurements from the same amount of cells as opposed to this study where the n values varied for each condition.

References:

Allaman, I., Gavillet, M., Bélanger, M., Laroche, T., Viertl, D., Lashuel, H. A., & Magistretti, P. J. (2010). Amyloid-β Aggregates Cause Alterations of Astrocytic Metabolic Phenotype: Impact on Neuronal Viability. *Journal of Neuroscience*, *30*(9). Retrieved from http://www.jneurosci.org/content/30/9/3326.short

Amyloid β-Protein Fragment 25-35 ≥97% (HPLC) | Sigma-Aldrich. Retrieved April 30, 2017, from http://www.sigmaaldrich.com/catalog/product/sigma/a4559?lang=en&region=US

Baracca, A., Sgarbi, G., Solaini, G., & Lenaz, G. (2003). Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F(0) during ATP synthesis. *Biochimica et Biophysica Acta*, *1606*(1–3), 137–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14507434

Black, S. E. (2017). Effects of intracellular beta amyloid on mitochondria in triturated glial cells of Gallus gallus. *Wheaton Journal of Neurobiology Research Wheaton Journal of Neurobiology Research BIO Neurobiology Wheaton College*, *324*. Retrieved from file:///C:/Users/Ryan J McKeon/Downloads/Black S final submission for WJNR Spring 2017 Intracellular BA effects on mitochondria in glia.pdf

Finder, V. H., & Glockshuber, R. (2007). Amyloid-β Aggregation. *Neurodegenerative Diseases*, *4*(1), 13–27. https://doi.org/10.1159/000100355

Goldman, B. (2013). Scientists reveal how beta-amyloid may cause Alzheimer's | News Center | Stanford Medicine. Retrieved from https://med.stanford.edu/news/all-news/2013/09/scientists-reveal-how-beta-amyloid-may-cause-alzheimers.html

Humure, C. (2017). Wheaton Journal of Neurobiology Research The effect of intracellular beta-amyloid on mitochondria activity as demonstrated through fluorescence microscopy The effect of intracellular beta-amyloid on mitochondria activity as demonstrated through fluorescence microscopy. *Wheaton Journal of Neurobiology Research BIO Neurobiology Wheaton College*, *324*. Retrieved from file:///C:/Users/Ryan J McKeon/Downloads/Humure C Final Submission for WJNR Spring 2017 intracellular BA and mitochondrial activity.pdf

Kann, O., & Kovács, R. (2007). Mitochondria and neuronal activity. *American Journal of Physiology - Cell Physiology*, *292*(2). Retrieved from http://ajpcell.physiology.org/content/292/2/C641.short

Moreira, P.I. Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. (2010). *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1802*(1), 2–10. https://doi.org/10.1016/J.BBADIS.2009.10.006

Modica-Napolitano, J. S., & Aprille, J. R. (2001). Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. *Advanced Drug Delivery Reviews*, *49*(1–2), 63–70. https://doi.org/10.1016/S0169-409X(01)00125-9

Morris, R. L. (2017). Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION. Retrieved October 2017, from http://icuc.wheatoncollege.edu/bio324/2015/NBlabPrimaryTissCultureProc1Dissn2015.htm

Murphy, M. P., & Levine, H. (2010). Alzheimer's disease and the amyloid-β peptide. Journal of Alzheimer's Disease. NIH Public Access. https://doi.org/10.3233/JAD-2010-1221

Nagele, R. G., Wegiel, J., Venkataraman, V., Imaki, H., Wang, K.-C., & Wegiel, J. (2004). Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiology of Aging*, *25*(5), 663–674. https://doi.org/10.1016/J.NEUROBIOLAGING.2004.01.007

Shevtzova, E. F., Kireeva, E. G., & Bachurin, S. O. (2001). Effect of β β β β β-Amyloid Peptide Fragment 25-35 on Nonselective Permeability of Mitochondria. Byulleten Eksperimentalnoi Biologii I Meditsiny, 132(12), 652–656.

Terman, A., & Brunk, U. T. (1998). Lipofuscin: mechanisms of formation and increase with age. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, *106*(2), 265–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9531959

Verburg, J., & Hollenbeck, P. J. (2009). Mitochondrial Membrane Potential in Axons Increases with Local NGF or Semaphorin Signaling. J Neurosci, 28(33), 8306–8315. https://doi.org/10.1523/JNEUROSCI.2614-08.2008.Mitochondrial

Experiment done in collaboration with Omar Raouf, Dan Southerland, Matt Morgan, Stephanie Martin, Luis Lazo