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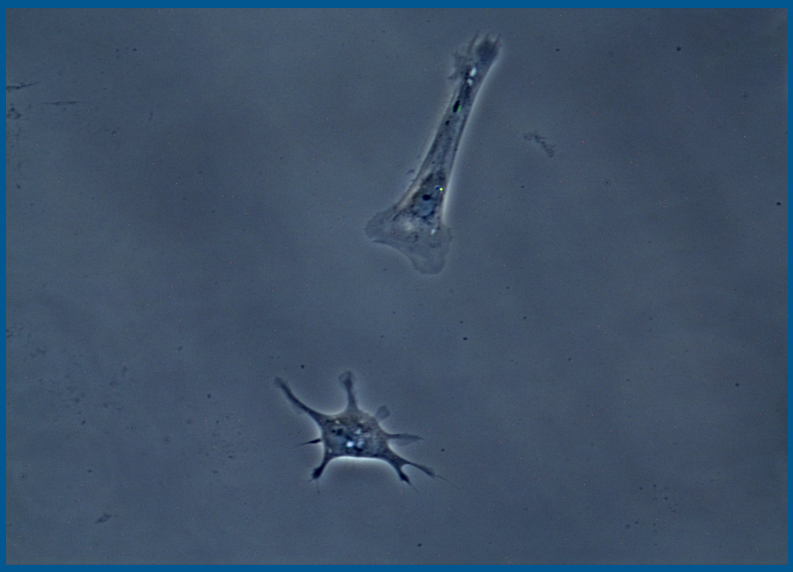

Effects of Amyloid β-Protein on autophagy in *Gallus gallus* embryonic peripheral glial cells as measured by lysosomal activity

Cynthia Mercedes

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Introduction

In Alzheimer's disease (AD), Beta-amyloid alongside tau tangles are the most common forms of synaptic disruptions that negatively impact proper neuronal functioning (Games et al., 1995). The amyloid beta precursor protein (APP) is a transmembrane protein present in neurons from which amyloid proteins are broken down. The amyloid beta proteins that are released from APP aggregate and become key components in plaque formation (Masliah et al.,1996). When these beta amyloids are released from the membrane, they clump together and form plaques in the extracellular space (Ekstrom, L., personal communication, August, 2014). These newly formed plaques are now harmful to the neuron. Beta amyloid plaques specifically interfere with regular neuron functions such as communication, as they block synaptic transmission by creating a blockage at the synaptic cleft (Ekstrom, L., personal communication, August, 2014). By hindering synaptic communication, neurons then die. The death of neurons further promotes the loss of neuronal communication throughout the brain, which further reduces cognitive functioning, and ultimately supports the progression of AD.

Glial cells in particular, are different from neurons. The main differentiating characteristics include their lack of axons and dendrites and their lack of participation in synaptic communication (Purves, 2001). Within the nervous system, glial cells hold specific roles. Glial cells are responsible for modulating synaptic activity, providing stability for neurons, insulate neurons, and destroy pathogens, aid in neural recovery and remove dead neurons (Purves, 2001). In addition, glial cells remove cellular debris from sites of injury (Purves, 2001). In AD specifically, one of the main functions of glial cells is to regulate the levels of beta amyloid levels within the brain (Ries, 2016).

Autophagy is the mechanism that removes unnecessary components or debris affecting the cell (Funderburk, 2010). It is a major cellular degradation pathway in which cells including neurons and glial cells remove damaged organelles and waste such as aggregated proteins. It is also a major pathway for organelle and protein turnover, and has been implicated in AD. (Boland, 2008) The autophagic pathway is responsible for sending debris and cellular waste to lysosomes, by fusing the autophagosomes to a lysosome, for degradation breakdown (Funderburk, 2010). However, research has shown a negative correlation between autophagy and neurodegeneration. A study showed that a dysfunction in this mechanism has been correlated with AD progression (Nilsson, 2013). Supporting this finding, a different study showed that altered lysosomal function contributed to the progression of neurodegeneration, resulting in an

accumulation of beta amyloid (Nixon, 2000). This current study specifically addresses the effects of Amyloid Beta Protein Fragment 25-35 on autophagy as measured by lysosomal activity. Based on previous studies, it is hypothesized that by introducing extracellular beta amyloid to glial cells, we would see a decrease in autophagy as beta amyloid alters autophagy function and increases cytotoxicity.

Materials and Methods

Materials

Amyloid Beta Protein Fragment 25-35, Cat No. A4559-1 MG purchased from Sigma.
Amyloid Beta Protein Fragment 35-25, Cat No. A2201- 250UG purchased from Sigma.
LysoTracker® Green DND-26, Cat No. 8783 purchased from Cell Signaling.
Nikon Eclipse E200 at 40x magnification, SPOT InSight Firewire, and the SPOT program, version 4.5.9.9, on a Macintosh computer- Tauro in the ICUC
Image J Version 1.51, on a Macintosh computer

Coverslip Preparation and Treatment, Dissection, and Cell Culture

Dissections and culture of 10-day-old *Gallus gallus* chick embryo neurons and glia were performed as per Dr. Morris' procedure (Morris, 2015a). Dorsal root ganglia (DRGs) and sympathetic nerve chains were specifically dissected and collected to be used as primary cultures for this study. Cell observations were done as described by Morris (2015b). After dissection, cells were plated and grown for 24 hours before experimental or control exposure.

Stock Solutions and Exposure

Control Stock Solution

The control exposure for this experiment consisted of DMSO solution in growth medium. Cells were exposed to 2 ml of growth medium and 50 ul of DMSO over a 24-hour incubation period in a 37C incubator. This control stock solution was a control for both the Amyloid Beta Protein Fragment 25-35 and Reverse 35-25 fragment. It also controlled for the added presence of external material.

Experimental Control- Amyloid Beta Protein Reverse Fragment 35-25 Stock Solution

The experimental control exposure for this experiment consisted of the Amyloid Beta Protein Reverse Fragment 35-25 in growth medium. The stock solution for the Amyloid Beta Protein Fragment 35-25 created by adding 94 uL of DMSO to the 250 ug of the Amyloid Beta Protein Fragment 35-25 powder purchased from Sigma to achieve a final stock solution concentration of 25uM, as this concentration was proven to be successful in previous studies (Yang, 1998). The working solutions for treatment were made by adding 20 uL of the created stock solution to 1 ml of growth medium. Cells in a petri dish were exposed to 1 ml of the working solution and 1 ml of growth medium over a 24-hour incubation period in a 37C incubator. This experimental control stock solution controlled for both the Amyloid Beta Protein Fragment 25-35 as the reverse fragment contained all of the same amino acids however is inactive in the reverse conformation.

Experimental Condition- Amyloid Beta Protein Fragment 25-35 Stock Solution

The experimental exposure for this experiment consisted of the Amyloid Beta Protein Fragment 25-35 in growth medium. The stock solution for the Amyloid Beta Protein Fragment 25-35 created by adding 377 uL of DMSO to the 1 mg of the Amyloid Beta Protein Fragment 25-35 powder purchased from Sigma to achieve a final stock solution concentration of 25uM. The working solutions for treatment were made by adding 20 uL of the created stock solution to

1 ml of growth medium. Cells in a petri dish were exposed to 1 ml of the working solution and 1 ml of growth medium over a 24-hour incubation period in a 37C incubator.

LysoTracker Solution

Stock solution and working solution were created following procedure provided by Keran Yang (Yang). Working solutions were made by diluting 1 uL of LysoTracker was diluted in 16.5 mL of HBSS.

LysoTracker Labeling

After 24 hours of Amyloid Beta Protein exposure, all growth mediums in petri dishes were removed. 1 mL of LysoTracker solution was placed in the petri dish, kept out of light, and was placed in the incubator, for a total of 8 minutes. This was done one petri dish at a time in order to prevent cell death or blebbing. Following the 8-minute incubation period, the LysoTracker was removed from the petri dishes and cells were washed three times with warm HBSS. The HBSS was warmed in a water bath at 37℃ for 15 minutes. Each wash was approximately 30 seconds each. Washes were executed around the perimeter of the coverslip to avoid removal of cells or damage to cells. Following the washes, 2 mL of fresh growth medium was added to each petri dish in preparation for coverslip mounting.

Data Collection and Analysis

Images of the glial cells were taken using a Nikon Eclipse E200 at 40x magnification. SPOT program version 4.5.9.9, on a Macintosh computer. Fluorescence images were collected with an exposure time of 20 seconds. Transmitted images as well as fluorescence images were captured and overlaid on ImageJ program, version 1.51 on a Macintosh computer. Lysosomal activity was measured with the LysoTracker by measuring the brightness of the bright green puncta depicted in the cells. Measurements were indicated by the integrated density measured on ImageJ, and parameters and thresholds were set on ImageJ following the protocol provided as follows (Fitzpatrick, 2017). Cells were selected on ImageJ using the drawing tool. Following, the "set measurements" option was selected under the Analyze option (Fitzpatrick, 2017). Then, "measure" was selected under the same Analyze option (Fitzpatrick, 2017). To create a background standard, a new region within the image with no fluorescence was selected (Fitzpatrick, 2017). This process was repeated for all cells and images selected for analysis. Once all data was selected for, the integrated density of each image including the background baseline was collected as raw data (Fitzpatrick, 2017). The corrected total cell fluorescence (CTCF) was then calculated as follows: "CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)" (Fitzpatrick, 2017). The calculated CTCF values were then averaged by condition. The average CTCF calculated per condition helped determine the condition with the most lysosomal activity, which was in turn, interpreted to mean the most autophagy.

Results

The results of this experiment refuted the hypothesis. Results showed higher autophagy after Amyloid Beta Protein Fragment 25-35 exposure, as opposed to the predicted decrease. Shown in Figures 1, 2, and 3, lysosomal activity is indicated by fluorescent puncta, on the left side of the figures. On the right side of the image, you can see where their fluorescence is indicated within the cell. Notice an increase in fluorescence from the DMSO control, to the reverse beta amyloid fragment, and to the forward amyloid fragment. Figure 4 further showcases this pattern as the forward beta amyloid fragment produced the greatest brightness average whereas the DMSO control showed the least, indicating an effect of beta amyloid exposure.

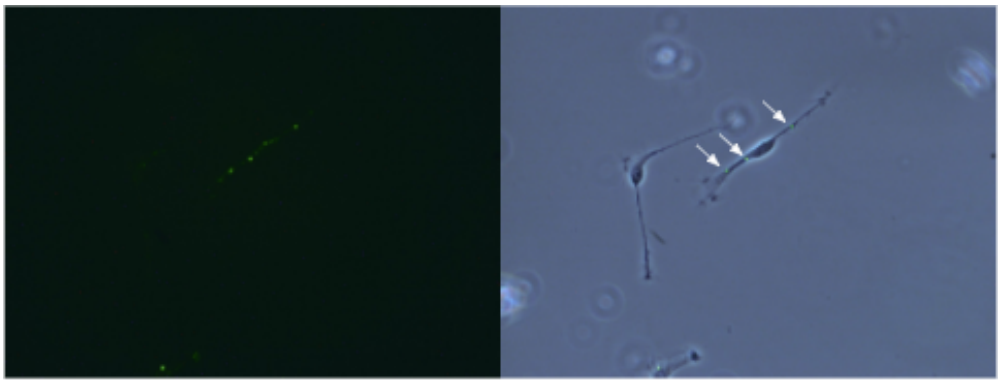

Figure 1. Lysosomal activity in *Gallus gallus* embryonic glial cell treated with DMSO. Fluorescence images on the left shows lysosomal activity as depicted by LysoTracker, labeled by green fluorescent puncta. Overlaid image on the right shows the fluorescent puncta on the glial cells indicated by the arrows.

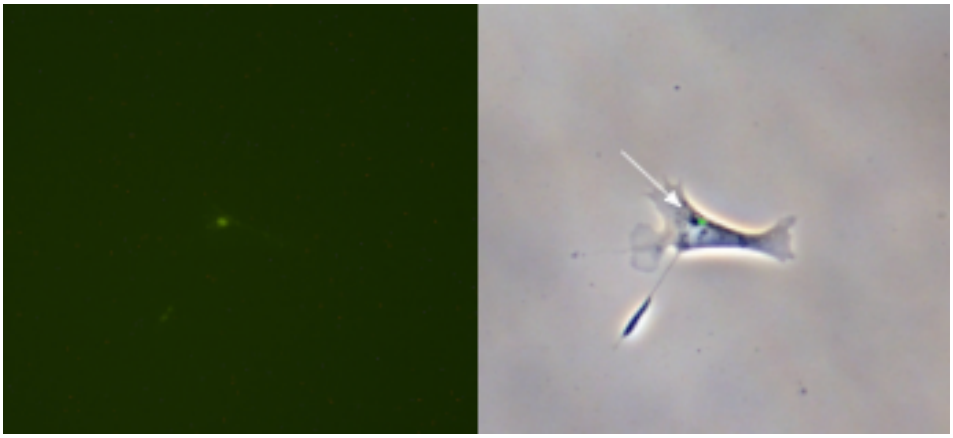

Figure 2. Lysosomal activity in *Gallus gallus* embryonic glial cell treated with 25mM Amyloid Beta Protein Reverse Fragment 35-25. Fluorescence image on the left shows lysosomal activity as depicted by LysoTracker, labeled by green fluorescent puncta. Overlaid image on the right shows the fluorescent puncta on the glial cells indicated by the arrows.

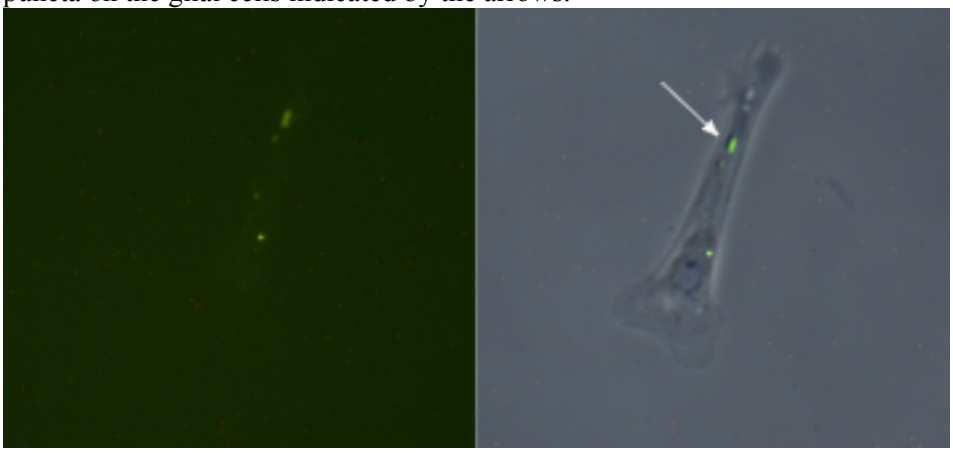

Figure 3. Lysosomal activity in *Gallus gallus* embryonic glial cell treated with 25mM Amyloid Beta Protein Fragment 25-35. Fluorescence image on the left shows lysosomal activity as depicted by LysoTracker, labeled by green fluorescent puncta. Overlaid image on the right shows the fluorescent puncta on the glial cells indicated by the arrows.

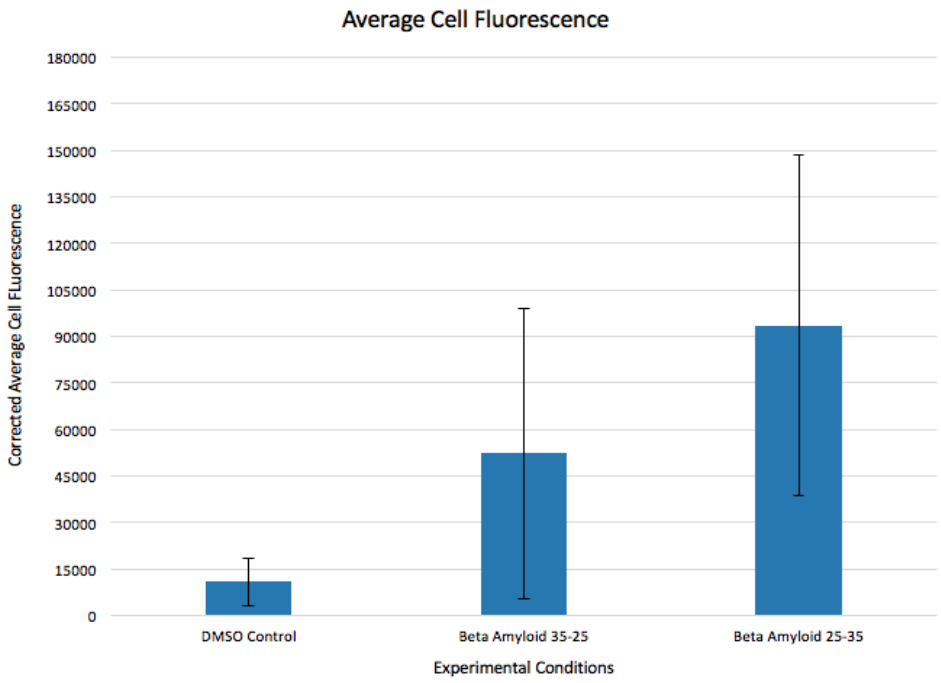

Figure 4. Average fluorescence in glial cells exposed to DMSO, Beta Amyloid 35-25 reverse fragment, and Beta Amyloid 25-35 fragment. For the DMSO condition, four integrated densities were averaged, for the reverse fragment, seven densities were averaged, and for the forward fragment, 11 densities were averaged. The DMSO control produced the lowest fluorescence, indicating by the least amount of lysosomal activity as labeled with LysoTracker. Beta Amyloid 25-35 fragment produced the greatest fluorescence, indicating the most lysosomal activity as labeled with LysoTracker. Indicated cell fluorescence was determined with ImageJ by measuring level of brightness as integrated density in comparison to a blank threshold.

Discussion

Based on previous research, it was hypothesized that autophagy would decrease in glial cells after exposure to extracellular beta amyloid. The results found in this study refute the hypothesis. As shown in Figures 1-3, autophagy was present in all cells post exposure to amyloid beta or DMSO, however in Figure 4 it is shown that there was a higher amount of autophagy detected in glial cells that were exposed to the active extracellular beta amyloid 25-35. Based on the average measured integrated density and CTCF across all experimental conditions, the Beta Amyloid 25-35 fragment had the highest average fluorescence (Figure 4). It is also important to note that although the reverse beta amyloid fragment 35-25 was an inactive form for the forward fragment, there was still a high presence of lysosomal activity detected by the LysoTracker. We believe this took place because protein fragments were introduced to the cell which further induced an automatic response within the cell to remove what the cell considered to be cellular debris. Although the fragment is an inactive form of beta amyloid, it might have still considered the fragments as a form of cellular waste.

The results found for this experiment contradict the studies described above however, a few different studies in fact support the results found. Although it is expected to see a decrease in autophagy within the cells infected with beta amyloid, as studies have proven that a dysfunction in autophagy correlates with the progression of AD, autophagosomes, lysosomes, and

autolysosomes are still present within the cells. This study was measuring lysosomal activity within the cells as measured with LysoTracker. What I believe the LysoTracker could have been labeling is the presence of lysosomes and lysosomal particles within the cells.

Research has shown that in AD, autophagy, specifically the activation of the endocytic pathway (part of the degradation pathway) is present in AD (Funderburk, 2010). However, certain areas within the brain showcase abnormalities in the endocytic pathway (Funderburk, 2010). These abnormalities include an increase in the size of endosomes present in the cell (Funderburk, 2010). These abnormalities indicate that although the function and efficiency of autophagy are disrupted and altered, autophagy is still present. Autophagy is a mechanism still active within the cell and lysosomes and autophagosomes are still present within the cell, although their efficiency in breaking down cellular debris is altered. This studies suggest that beta amyloid protein aggregates are still engulfed by these organelles and vacuoles; however, they are not being properly degraded. What we believe the LysoTracker may have been labeling is the increase in presence of these vacuoles within the cells as beta amyloid was presented, not the breakdown of beta amyloid as implied by studies that supported the hypothesis of decreasing autophagy.

A different study also showed that oxidative stress within the cell enhanced macroautophagy and in turn lead to intralysosomal accumulation of beta amyloid (Zheng, 2009). In addition, the increase in macroautophagy produced more toxicity within cells (Zheng, 2009). Although altered autophagy within the cell did not appropriately degrade beta amyloid and further induced cytotoxicity within the cell, the activation was still present and we believe that it would have been detected by the LysoTracker, if autophagy was being labeled.

We realize that this study was testing for a change in autophagy activity, specifically the change in presence of lysosome and autophagosomes. Although the efficiency of debris degradation may have not been detected, as this is not within the scope of the study, we believe that the increase in fluorescence labeling was due to beta amyloid inducing autophagy.

Limitations within the experiment included sample size, exposure time, and beta amyloid doses. By increasing the amount of trials tested, increasing the amount of cells tested per condition, and by testing a variety of beta amyloid doses and exposure times, we may be able to establish a clear understanding for the effects of beta amyloid on autophagy, further increasing the power of the study. In addition, by eliminating human error through out the experiment, including cell handling and inconsistent drug exposure methods, one can eliminate variability within the results.

Overall, this study brings us closer to understanding the cellular mechanisms that protects adequate cell functioning. Although autophagy is important for cellular debris degeneration, it is in fact a major hallmark in AD. By understanding the mechanism by which autophagy is impacted by beta amyloid and vice versa, we will be one step closer in understanding how we can improve this protective mechanism to there reduce the progression of AD and other neurodegenerative diseases.

References

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H., & Nixon, R. A. (2008). Autophagy Induction and Autophagosome Clearance in Neurons: Relationship to Autophagic Pathology in Alzheimer's Disease. *Journal of Neuroscience,28*(27), 6926-6937. doi:10.1523/jneurosci.0800-08.2008

Fitzpatrick, M. (n.d.). Measuring cell fluorescence using ImageJ¶. Retrieved April 18, 2017, from http://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html

Funderburk, S. F., Marcellino, B. K., & Yue, Z. (2010). Cell "Self-Eating" (Autophagy) Mechanism in Alzheimer's Disease. *Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine,77*(1), 59-68. doi:10.1002/msj.20161

Zheng, L., Kågedal, K., Dehvari, N., Benedikz, E., Cowburn, R., Marcusson, J., & Terman, A. (2009). Oxidative stress induces macroautophagy of amyloid β-protein and ensuing apoptosis. *Free Radical Biology and Medicine,46*(3), 422-429. doi:10.1016/j.freeradbiomed.2008.10.043

Masliah, E., Sisk, A., Mallory, M., Mucke, L., Schenk, D., & Games, D. (1996). Comparison of Neurodegenerative Pathology in Transgenic Mice Overexpressing V717F b-Amyloid Precursor Protein and Alzheimer's Disease. *Journal of Neuroscience,16*(18), 5795-5811.

Morris, R.L. (2015a) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 1: dissection. Accessed <insert date you accessed article here>. Available at: http://icuc.wheatoncollege.edu/bio324/2015/NBlabPrimaryTissCultureProc1Dissn2015.htm

Morris, R.L. (2015b) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 2: observation of live unlabled cells. Accessed 11/04/2015. Available at: http://icuc.wheatoncollege.edu/bio324/2015/NBlabPrimaryTissCultureProc2ObservnOfLliveUnlabeledCells2015.htm

Nixon, R. A., Cataldo, A. M., & M, P., Mathews. (2000). The Endosomal-Lysosomal System of Neurons in Alzheimer's Disease Pathogenesis: A Review. *Neurochemical Research,25*, 1161-1172.

Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., . . . Saido, T. (2013). Aβ Secretion and Plaque Formation Depend on Autophagy. *Cell Reports,5*(1), 61-69. doi:10.1016/j.celrep.2013.08.042

Novakovic, Biljana, Collaborator

Purves, D., & Williams, S. M. (2001). *Neuroscience. 2nd edition*. Sinauer Associates.

Ries, M., & Sastre, M. (2016). Mechanisms of Aβ Clearance and Degradation by Glial Cells. *Frontiers in Aging Neuroscience,8*. doi:10.3389/fnagi.2016.00160

Wang, Yong, Lili Liu, Weimin Hu, and Guanglai Li. "Mechanism of soluble beta-amyloid 25–35 neurotoxicity in primary cultured rat cortical neurons." *Neuroscience Letters*618 (2016): 72-76. Web.

Yang, A. J., Chandswangbhuvana, D., Margol, L., & Glabe, C. G. (1998). Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid AB1-42 pathogenesis. Journal of Neuroscience Research, 52(6), 691-698. doi:10.1002/(sici)1097-4547(19980615)52:6<691::aid-jnr8>3.0.co;2-3

Yang, Keran, Collaborator

Zheng, L., Kågedal, K., Dehvari, N., Benedikz, E., Cowburn, R., Marcusson, J., & Terman, A. (2009). Oxidative stress induces macroautophagy of amyloid β-protein and ensuing apoptosis. *Free Radical Biology and Medicine,46*(3), 422-429. doi:10.1016/j.freeradbiomed.2008.10.043