Amount of glia may decrease the abundance of mitochondria in axons

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Introduction
Each year approximately over 10 million people suffer globally from neurodegenerative diseases (Spuch, 2012). Alzheimer’s disease (AD) is the most common neurodegenerative disease and it affects approximately one-eighth of people older than 65 years old (Kandel, 2000). Five million people in the United States currently suffer from dementia, and with an increasing number of elderly people the population at risk for AD are increasing substantially (Kandel, 2000). Alzheimer’s can affect the loss of recent memories, problems with language, abstract thinking, trouble knowing time place and judgment. AD is characterized by three dramatic abnormalities of the brain (Alzheimer’s Association, 2017). The presence of enlarged ventricles, extracellular plaques called amyloid, which are large aggregates of fibrillar peptides arranged as sheets, and cytoskeletal abnormalities such as the accumulation of neurofibrillary tangles called tau tangles (Alzheimer’s Association, 2017). AD comes in many varying degrees of the disease but there are two hallmark pathologies required for a diagnosis of Alzheimer’s disease, which are the extracellular plaque deposits of the β-amyloid peptide (Aβ) and the neurofibrillary tangles of the microtubule binding protein tau (Murphy, 2010).

Abnormal behavior such as hyper phosphorylation of tau proteins causes this microtubule-binding protein to come apart from its original structure and create tau tangles in neurons (Alzheimer’s Association, 2017). As a result, microtubules that were once held together by tau are disassembled. Microtubules are crucial for the formation of axons and the transportation of materials and organelles like mitochondria through the axon (Kandel, 2000). Beta-amyloid plaques also contribute to the Alzheimer’s effects by aggregating within the cell and inducing cell death (Murphy, 2010). Beta-amyloid present in cells are known to have adverse effects on their organelles. Amyloid beta and amyloid-precursor protein are known to localize to mitochondrial membranes, block the transport of nuclear-encoded mitochondrial proteins to mitochondria, interact with mitochondrial proteins, disrupt the electron-transport chain, increase reactive oxygen species production, cause mitochondrial damage and prevent neurons from functioning normally (Reddy et al 2008). In AD neurons, both anterograde and retrograde transport of mitochondria are slow because of the presence of a substantial number of defective and functionally inactive mitochondria (Reddy et al 2010). The fact that the transport of mitochondria is being affected is crucial because the mitochondria is a fundamental organelle for the cell and is required for the cell to keep functioning and perform all its tasks. Although multiple cellular changes have been reported to be involved in AD pathogenesis, synaptic
pathology and mitochondrial oxidative damage and oxidative stress in particular play a critical role in the pathogenesis of AD and is intimately linked to aging, the best established risk factor for AD (Spuch, 2012). “It is generally accepted that an accumulation of Aβ in synapses and in synaptic mitochondria, particularly in neurons affected by AD, cause synaptic degeneration and cognitive decline in AD patients” (Reddy et al 2010). This is important to understand because synaptic degeneration is detrimental to the functioning of the cell, without a properly functioning synapse the neurons can’t transmit signals to the other cell making signal processes in the brain not possible. As of now the consensus on synaptic degradation is that it isn’t possible to restore the synapse once it has been degraded because of the limited regenerative capabilities (Dzamba et al 2016) of the neuron, therefore it relies and glial cells to protect it.

Another type of cell that is also affected by beta-amyloid and tau are glial cells. Glial cells are the supporting cells in the central nervous system and are responsible for supporting the maintenance of the neuronal cells (Nagele, 2004). Beta-amyloid also causes cell death in glial cells not just neurons. Because glial cells are also being destroyed the neurons that survived are unable to have the assistance of the nearby glial cells to support its growth, which may be another risk factor that increases the chance of neuron susceptibility to cell death (Nagele, 2004). “Glial cells are fundamental for the progression and outcome of AD either because they function as effector cells releasing cytokines that play a role in neuroprotection, or because they fail to fulfill their homeostatic functions, ultimately leaving neurons to face excitotoxicity and oxidative stress” (Dzamba et al 2016). Another risk factor for neurodegeneration may be “Activated microglia also release excessive quantities of glutamate, thereby inducing excitotoxicity and neurodegeneration” (Takeuchi, 2010) and this contradicts the glial homeostatic function. With glial cells failing to fulfill their homeostatic functions, glia plays a role in making sure the neuron can function properly by being healthy and thereby having healthy functioning mitochondria.

In this experiment, I will test the hypothesis that the amount of glia present will decrease the mitochondrial abundance in neuronal axons. I theorize that if the presence of glial cells is necessary to maintain the high abundance of mitochondria in neurons then the loss of glial cells would cause a decrease in abundance of mitochondria in neurons. This experiment will be using Gallus Gallus sympathetic neurons for sparse cell cultures. The control for this experiment will be sparse culture cells composed of neurons and glia with nothing done to it. The experimental cultures will be treated with a dissociation procedure, derived from (Jana et al 2007), to separate the glial cells from the neuronal cells. Finally, Rhodamine 123, which is a fluorescent molecule, will be used to stain mitochondria and fluorescent microscopy to visualize the mitochondria and take still images of neuronal axons. This is interesting because mitochondria are an important organelle in the cell and plays a vital role in synthesizing energy for the cell which is crucial because of the high energy demand and limited regenerative capability (Nagele, 2004), any improper functioning of mitochondria can have devastating effects on neuronal survival. “There is ample evidence of impaired mitochondrial function as a cause rather than consequence of neurodegeneration” (Beal et al 2012). Therefore, mitochondria are easy targets to affect the overall function of the cell. It is important to understand and study how effective the glial cells around the neurons contribute to the abundance of the mitochondria in neurons.

Materials
To conduct this experiment a separation technique derived from (Jana et al, 2007) was used on experimental cell cultures and Rhodamine 123 dye was used to label mitochondria in all cell cultures. The reagent used, rhodamine 123, was acquired from Sigma Aldrich catalog
number 62669-70-9. For imaging a Nikon Eclipse E400 phase microscope, with a Spot Insight Firewire 2 Megapixel camera attached by a 1.0x Nikon camera mount was used. Imaging was carried out using SPOT software version 5.2.5 on the iMac desktop computer running OSX 10.10.5 in the ICUC laboratory. Images were taken at 40x magnification and a Phase 2 lens. Software ImageJ on the iMac desktop computer in ICUC laboratory was used to quantify data.

**Methods**

**Dissection**

This experiment was done by using 10-day old *Gallus Gallus* embryos. Procedure was performed as described in the protocol of (Morris, 2015a). A total of 2 sympathetic nerve chains were collected. Sympathetic nerve chains were collected because they contain a higher number of neurons than dorsal root ganglions.

**Trypsinization**

After the dissection and the collection of the sympathetic nerve chains they were incubated at 37°C for 20 minutes with 1mL of trypsin. Then a full exchange was done with the trypsin and Hank’s balanced salt solution (HBSS) was added into the dish, to then triturate the sympathetic nerve chains to dissociate the cells. Must be careful to not destroy the cells if trituration was done too harshly the cells will be torn apart and not be useful for the experiment.

**Trituration**

Once triturated, sparse cultures containing neurons and glia from the sympathetic nerve chains were set out on 3 petri dishes. Each containing 1.5 mL of growth medium to prevent the cells from dying. The cells would be placed in the dishes with the growth medium with poly lysine cover slips in them. The first dish would be left alone and be the control while the other two dishes undergo the rest of the dissociation procedure. Allow the dishes to sit still for a total of 22 minutes and then after the 22 minutes take one of the dishes and gently shake them around in a circular motion for 2 minutes to help sever the connections of cells that didn’t adhere to the treated cover slips, procedure derived and based from protocol of (Jana et al, 2007). Collaboration was done with Lena O’Flynn. After 2 minutes the solution was decanted from the dish and placed into the fourth dish and new growth medium was added to the empty dish. The same process was repeated for the 3rd dish and then finally 4 cell cultures are left.

After 48 hours cells were looked at under an inverted microscope using the 4x magnification coverslips were scanned through in order to quantify the number of neurons that grew, and the number of glial cells present to later calculate the glia to neuron ratio. Firstly the definition of glia and neurons had to be established to properly quantify them. Neurons were classified as cells with spherical cell bodies with axonal projections coming out from it. Glial cells are classified as polygonal shaped cell with no projections coming from it. The same process was repeated for the rest of the other cultures.

**Rhodamine 123 Preparation and Cell Staining**

4 cell cultures were used for this experiment therefore 4mL of rhodamine 123 working solution was needed. A 1:1000 dilution was prepared by adding 4μL of rhodamine 123 into 4mL of growth medium. Dishes required a 1:1000 working solution of rhodamine 123 and a full exchange of HBSS from the petri dish and added 1mL of rhodamine 123 covered with aluminum foil and let incubate at 37°C for 10 minutes. The rhodamine 123 solution was then carefully
pipetted out and 3 washes were performed on the cells using HBSS to rid of excess Rhodamine 123 and finally add 1mL of growth medium back into the solution. This section was then repeated for the rest of the dishes. This procedure was derived from the protocol (Sapiente, 2017) this section was done in collaboration with Arianna Persarik, Omar Raouf, Ryan McKeon, Matt Morgan, Stephanie Martin, Daniel Southerland.

**Chip chambers and data collection**

After cell staining chip chambers were used to be able to capture fluorescence images of mitochondria within axons. Chip chambers were created following the published protocol of Morris (2015b). Once cells were in chip chambers the cells were located using transmitted light on 10x magnification using Phase 1 lens and then magnification 40x and phase 2 lens were used to get a closer and clearer image on neuronal axon. A Nikon Eclipse fluorescence microscope with an objective lens of 40X PH2 was used to capture images of the cells observed in the ICUC lab. An exposure time of 5,000-8,000 milliseconds was used to capture fluorescent images of the reagent in mitochondria. Mitochondria were defined as figures that looked oval shaped and can be elongated. The program ImageJ was used to analyze the data for the abundance of mitochondria in neuronal axons in each of the images for each cell culture. This was done by opening the fluorescence image in ImageJ and use the marker tool to flag the individual mitochondria present in the axon. Later, using ImageJ again the line tool was used measure a length of 100 pixels and count the number of mitochondria per 100 pixels of axon. The data was then averaged together to get an average number of mitochondria per 100 pixels of axon in that image.

**Results**

Figure 1 shows mitochondria labeled by the Rhodamine 123 as a bright fluorescent green color. The mitochondria were counted using ImageJ then the data were normalized by calculating the average number of mitochondria per 100 pixels. The images shown are images of fluorescence images of the cells. The two graphs are there to demonstrate the ratio of neuron to glia in each cell culture both experimental and control dishes. Figure 4 shows the average number of mitochondria per 100 pixels of axon. Data were derived from quantification of mitochondria in axons from neurons in 12 images captured from control condition. Data were derived from quantification of mitochondria in axons from neurons in 46 images captured from experimental conditions.
Figure 1: Image of a neuron from experimental culture with its axons projecting out in three separate directions. Image was taken using fluorescent microscopy using the Nikon Eclipse camera at 40x magnification PH2. Image on the right shows the use of ImageJ software to quantify mitochondria in axons. This image is used to demonstrate the how quantification of mitochondria was done.

Figure 2: Image from control (left) and experimental (right) culture used to calculate the ratio of neurons to glia in control. Similar pictures were used to find the ratio of neuron to glia in experimental cultures. These images show how the control and experimental dishes looked like and the difference in number of glia and neurons after the experimental treatment.
Figure 3: Demonstrates the ratio of neurons to glia present in each culture. Ratio of neurons to glia in the control = 1 to 2.4, dish 2 = 2 to 0.7, dish 3 = 2.7 to 1, dish 4 = 2.6 to 1.1. Of all these the control had the greatest number of glia present and experimental 1 had the least number of glia present. Data were derived from quantification of neurons and glia by scanning the coverslip using a 4x magnification.

Figure 4: Chart shows the average number of mitochondria present in neuronal axons per 100 pixels. Control (dish 1) = 4.21, experimental (dish 2) = 3.3, experimental (dish 3) = 2.93, experimental (dish 4) = 2.81. Data were derived from quantification of mitochondria in axons from neurons in 46 images captured from experimental conditions and 12 images captured from control conditions.
Discussion

After analyzing the data, the data collected supported the hypothesis that the amount of glia present in a cell culture would decrease the abundance of mitochondria present in neuronal axons. Dishes 2, 3, and 4 were experimental dishes, which contained less glia. The data demonstrates that on average there are less mitochondria present per 100 pixels of axons in the dissociated cell cultures than there were in the control cell cultures. A possible explanation for this outcome may be because in the experimental trials the lesser presence of glial cells in culture compared to the regular presence of glial cells in control the experimental neurons don’t receive that added support from the glia to have a greater mitochondrial abundance. Therefore, suggesting that the presence of glial cells is essential for the strong development of neuronal cells including the abundance of mitochondria present in neurons.

To refine this experiment more samples would be needed to collect more data. With a small sample size, the amount of data collected could not have been enough. To gather stronger data a larger sample size would be needed, including more control cultures. Another source of variability would be that not enough images were taken or there was an uneven number of images taken from each cell culture that could have affected the data. To prevent this the same number of images will be taken from each cell culture. In addition, the images would need to be of similar cell structures, meaning that the images taken will try to match each of the images to make the data more reliable.

Future experiments to extend the results in a new direction would be to include the addition of beta amyloid fragments or tau proteins to test if absence of glia allows for greater or lesser affects from the beta amyloid or tau regarding mitochondrial abundance. There is currently a growing body of evidence to suggest that glial cells have a role in the pathology of Alzheimer’s Disease (Cabezas, 2014), and understanding their role would be a significant step towards understanding the mechanism of Alzheimer’s Disease. Other scientific research that would suggest that this may be something interesting to study as there has been recent interest regarding the intracellular mechanism of amyloid-beta and beta amyloid precursor protein which has made researchers consider a possibility that mitochondria-associated and mitochondrial amyloid-beta may have direct effects on neurotoxicity (Spuch, 2012).

References


I have abided by the Wheaton College honor code in this work.

-Luis Lazo

Collaboration was done with Arianna Persarik, Omar Raouf, Ryan McKeon, Matt Morgan, Stephanie Martin, Daniel Southerland, Lena O’Flynn.