A preliminary study on the effects of exogenous cholesterol on endocytic activity in living sympathetic neurons dissected from *Gallus gallus*

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Introduction

Endocytosis is the processes by which a cell’s plasma membrane folds on itself to take up extracellular material such as macromolecules and particulate substances as stated by Alberts, Johnson and Lewis (2002). Endocytic activity in this report will focus primarily on pinocytosis, a type of endocytosis. Endocytosis is essential in neuronal cells as neurotransmitters are recycled from the extracellular environment highlighting the importance of the plasma membrane in cells. Plasma membranes are essentially the gateway to the cell that have a crucial role in development, neurotransmission, intracellular communication, signal transduction and cellular and organism homeostasis as stated by Conner and Schmid (2003).

It has become increasingly clear that the molecular interactions within the plasma membrane are tightly coordinated with cell physiology (Conner, Sean D. Schmid, Sandra L., 2003). Moreover, alterations of the endocytic pathway have been attributed a crucial role in the pathophysiology of certain neurological diseases (Parton, R. G. and Dotti, C. G., 1993). Di Paolo and Kim have found that cholesterol is increasingly linked to Alzheimer’s disease pathology (2011) and a 30% increase in membrane cholesterol has been observed by Marquer et al.’s study (2014) in brain samples from deceased individuals with Alzheimer’s disease. (Marquer, Catherine et al., 2017). In the current preliminary study, we tested the hypothesis that an increase in transient membrane cholesterol will affect endocytic activity of neuronal cells. I chose this hypothesis, as it remains unclear whether sporadic Alzheimer’s disease could be initiated in cells due to the disruption of cholesterol metabolism leading to a change in neuronal membrane cholesterol. While most Alzheimer’s disease research is focused on the formation of amyloid plaques, understanding the link between cholesterol and Alzheimer’s phenotypes may provide us with an alternative pathophysiological explanation to the disease.

The process of endocytosis begins with an initial invagination of the plasma membrane, which pinches off into the cytosol of the cell forming an endocytic vesicle containing the ingested material (Alberts et al., 2002). Pinocytosis is distinguished by the size of the endocytic vesicle formed as they are generally 100nm in diameter (2002) and are rapidly targeted to a distinct membrane bound endocytic organelle known as an early endosome (Jovic, Marko et al., 2010). Endosome enlargement has been reported as an early phenotype of sporadic Alzheimer’s Disease and it has been observed that an increase in membrane cholesterol resulted in increased early endosome sizes and aggregation in Rattus rattus neurons (Marquer et al., 2014). The neuronal cells used in the current study were dissected from 10-day old chick embryos (Gallus gallus) as they provide a good model system for studying neurons because of
their well-documented development cycle and easy dissection based on Darnel and Schoenwolfs’ developmental biology protocols for the chick embryo (2002). Sympathetic nerve chains and dorsal root ganglia were harvested from the chick embryos peripheral nervous system. In this study, I treated 5-day old neuronal cells in primary culture with a methyl beta cyclodextrin (MβCD) cholesterol complex, which can deliver cholesterol directly to the plasma membrane in neurons (Marquer, Catherine et al., 2017). After cholesterol modulation, I analyzed endocytic activity by observing and comparing the cellular phenotypes correlated with Alzheimer’s disease.

**Materials and Methods**

**Coverslip Sterilization and Treatment**

22x22 mm coverslips were sterilized as per Morris’ protocol in “Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection” (2017) prior to treating one side of coverslip with poly-L-lysine and laminin.

**Dissection & Primary Cell Culture**

Dorsal root ganglia (DRG) and sympathetic nerve chains were dissected and cultured in a modified Leibovitz-15 (L-15) medium and kept in a low CO₂ incubator at 37°C as described by Morris (2017). Neuronal cells were cultured on coverslips treated with poly-L-lysine and laminin as per protocol in “Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection” (Morris, 2017).

**MβCD-Cholesterol Stock Solution**

The MβCD-Cholesterol was obtained from Sigma-Arich (C4951) and dissolved in Hanks balanced salt solution (HBSS). 18.4 mg of the MβCD-Cholesterol was added to 9.2 ml of HBSS to make a stock solution of 2 mg/ml. The stock solution was prepared in HBSS because it is a polar solution that does not perturb osmotic potential of neurons.

**Cholesterol Modulation**

A 1:5 and 1:10 dilution with HBSS was performed on the 2 mg/ml stock solution to make 200 μg/ml and 400 μg/ml solutions followed by half-exchanges with sparse cell cultures to attain final working solutions of 100 μg/ml and 200 μg/ml in culture. The cholesterol will be at twice the desired concentration until the 1:1 dilution with the growth medium, bovine serum albumin (BSA), to attain working concentrations. The neurons were incubated at 37°C for 30 minutes. The assigned working solution concentrations and methods obtained as per protocol of Riff, Callahan and Sherman (2005) in “Cholesterol-Enriched Membrane Microdomains”. After incubation, cultures were washed with growth medium and stayed in growth medium.

**Chip Chambers**

Live cells were observed in chip chambers made as per protocol for Morris (2017). One chip chamber was made for each of the studied groups of neuronal cells; control, 100 μg/ml MβCD-Cholesterol and 200 μg/ml MβCD-Cholesterol.
Observation of Neuronal Cells: Microscopy & Imaging

Neuronal cells were observed in their corresponding chip chambers with a Nikon Eclipse E200 phase microscope at 40x magnification and the Phase 2 lens to properly locate and identify endocytic vesicles. A Sony DFW-X700 Digital Interface with a X1 magnification was used. Chip chambers were kept at 37°C with a LASKO Ceramic Air Heater, model: 754200, and a small thermometer placed by the chip chamber. The imaging was carried out using BTV software on a 2013 iMac (Gemini) computer in the Imaging Center for Undergraduate Collaboration (ICUC) at Wheaton College in Norton, MA. A screenshot of the live feed of the neuronal cells was taken every 10 seconds for 3 minutes for each of the chip chambers.

Data Analysis: Quantification

Images were processed using ImageJ software version 1.8.0_66. The images of the control and experimental group were cropped around the perimeter of the cell of focus using the Freehand Selection Tool on ImageJ to attain the size of the cell in pixels^2. To quantify endosomes, I used Process>Find Maxima on ImageJ for an estimate to the number of endosomes in a random area within the cell. To do so I used Edit>Selection>Specify and designated an area within the neuronal cell of study, an oval, with a width and height of 150 pixels (17671.46 pix^2). The X coordinate and Y coordinates for the oval varied between experimental groups and control as the studied cells were in different locations on the screen but the area of the oval remained consistent. Noise Tolerance was set to 8 to account for the resolution of the images for both the control and experimental groups. Count was selected for the Output Type. Light Background, Exclude Edge Maxima and Preview Point Selection were chosen to display the number of endosomes counted. Potential intracellular vesicles and endosomes had a higher contrast compared to the background image. For my experiment, I have defined the parameters at which I identify endosomes as points with higher contrast for both my experimental groups and control. There is no evidence that the structures identified are indeed endosomes but based To quantify the sizes of endosomes, the ranges of pixel diameter were taken for the largest and smallest contrast points that moved throughout the time-lapse. The data was collected and processed using Microsoft Excel 2017. Data are derived from number of high contrast spots defined as intracellular endosomes in neuronal soma from 3 neurons with 18 images captured from 1 trial for the control. Further analysis on number of high contrast spots was performed to exclude erroneous structures that are not endosomes based on the size. Contrast points picked up by the software that were greater than 20 pixels in diameter were excluded from the endosome count.
**Results**

The size range of endosomes increased as cholesterol concentration increased as seen in Figure 2. This is noted as both the shortest and longest diameter in our cholesterol groups are greater than that of our control. The average number of endosomes per the $17671 \text{ pix}^2$ oval was highest for our $100 \mu g/ml$ cholesterol culture and lowest for the $200 \mu g/ml$ cholesterol culture as seen in Figure 3. Figure 1 contains still images on neurons before and after endosome count to highlight endosomes in cells.

*Figure 1* Neuronal Cells on the left show the fixed area that was observed represented by the oval. The neuronal cells on the right show how ImageJ marks areas with highest contrast which represent endosomes. Images were taken under 40X magnification with a Phase 2 lens every 10 seconds for 3 minutes.
Figure 2 The longest and shortest suspected endosomes were measure in diameter in pixels using the tracing tool on ImageJ.

Figure 3 Averages for suspected endosome per neuronal soma per culture.
Discussion and Conclusions

Data obtained from this preliminary study may support the initial hypothesis that an increase in transient membrane cholesterol will affect endocytic activity but method of data analysis should include a molecular tracker such as FITC-Dextran to ensure the presence of endosomes. Neurons treated with exogenous MβCD-Cholesterol were observed to have much larger ranges for suspected endosome diameter as seen in figure 2. This is in accordance with the findings of Marquer et al. (2017) that showed that an increase in transient membrane cholesterol resulted in endosomal enlargement, as in early sporadic Alzheimer’s Disease.

While the data possibly supports with the hypothesis, we were unable to verify the cholesterol content of the plasma membranes in the treated neurons. This step is crucial as we are attempting to replicate early Alzheimer’s disease phenotypes because those affected with the disease have a 30% increase in brain cholesterol which is believed to play a role in the progression and severity of the disease (Marquer et al. 2017). The data however does suggest that cholesterol modulation was successful as there is significant variability in the data between controls and experimental groups as seen in Figures 2 and 3. There was no statistical difference in the data as only one trial was performed for the control and the experimental conditions.

Alzheimer’s disease progressively worsens over time with an accumulation of senile amyloid plaques and neurofibrillary tau tangles as the two main lesions for explaining Alzheimer’s disease pathology as stated by Perl (2010). Little is known about the role that cholesterol plays in the formation of plaques and tangles in neuronal cells of Alzheimer’s afflicted individuals but Marquer has shown that stimulation cholesterol synthesis in mouse models overexpressing amyloid induces neurofibrillary tangle formation and tau phosphorylation (2014) highlighting the possible correlation between cholesterol increase and triggering of tau and amyloid pathologies. The current evidence provided by Barbero-Camps and Fernandez (2013) indicates that excess brain control regulates amyloid-B deposition, which can regulate cholesterol homeostasis. These studies show the significance in using cholesterol modulation as a method of testing for phenotypic Alzheimer’s disease pathology in neurons and can provide insight into possible treatments to regulate cholesterol metabolism in early stages of the disease.

However, there is limited to no evidence suggesting that phase contrasts correspond to endosomes in the cell. While some of the detected spots can be endosomes there is no way to know for sure which is why future studies should include the use of a tracker molecule. The neurons in culture are constantly endocytosing material from the exterior to the interior of the cell and if a tracker molecule is pinocytosis, then endocytic activity can be measured more effectively by observing the fluorescence of the cell. Future studies should include using either lysotracker to label endosomes or a fluorescent protein complex such as FITC-Dextran. The tracker molecule can be places in the culture medium and would be engulfed by the cell through pinocytosis (Alberts, 2002) and appear as bright spots with fluorescence microscopy. The data may then be quantified in a way that would show endocytic activity of the cell.
References


Morris, R. L. (2017). Problem Set 1 (Growth Medium Ingredients)

