Introduction:

Gliarial cells have a number of different vital functions within the nervous system. In particular, microglia help to maintain the nervous system’s immunity and macroglia aid in signal conduction through axons by enveloping the axons in myelin (Kandel et al., 2013). Without mitochondria, however, glial cells would not be able to operate due to the fact that mitochondria produce the energy currency that cells run on, known as adenosine triphosphate or ATP (Kandel et al., 2013). In addition, mitochondrial motility is essential due to the need for ATP throughout the entirety of a glial cell (Kandel et al., 2013). As is the case in axons, mitochondrial motility in glial cells is possible through the use of various motor proteins, including kinesins and dynein, which transport mitochondria along microtubules and actin filaments (Hollenbeck & Saxton, 2005; Morris & Hollenbeck, 1995). Recent studies have prompted questions regarding the possibility that the activity of glial cells and their mitochondria may be affected by mercury exposure.

It is not uncommon for humans to experience exposure to mercury through, for example, the consumption of fish, repair of a cavity with dental amalgam, or use of mercury-containing light bulbs (“Mercury and health,” 2013). The effects of mercury on the neurons and glial cells of the nervous system have been a concern for decades. However, only recently has significant progress been made on the topic. It has been thought that mercury and other heavy metal ions may interfere with mitochondrial participation in cellular respiration (Passow and Rothstein, 1960). More recently, Araragi et al. found that mercury-containing compounds prompt the mitochondria to release a compound called cytochrome c (Cyt c) into the cytosol (2003). The presence of Cyt c in the cytosol causes the activation of caspase-3 and -9, which are enzymes that induce apoptosis (Araragi et al., 2003). This process was observed in human and mouse cells (Araragi et al., 2003). In order to gain further perspective on the previously mentioned topics, this study examines the effects of mercury on the velocity of mitochondria within glial cells.

The organism used throughout this study is Gallus gallus, the domestic chicken. G. gallus is commonly used as a model organism and has been for over 100 years largely due to the vast information we currently hold with respect to the organism and its genome (Burt, 2007). For the purposes of this experiment, G. gallus is advantageous due to its brief development time (Morris, 2015a). A fertilized G. gallus egg only requires 10 days before the chick embryo is developed to a point at which constituents of the chick’s peripheral nervous system (dorsal root ganglia and sympathetic nerve chains) can be identified and removed (Morris, 2015a).
The aforementioned research suggests that compounds containing mercury negatively impact entire cells by inducing apoptosis and potentially by interfering with cellular respiration (Araragi et al., 2003; Passow and Rothstein, 1960). It is possible that this fatal impact on cells as a result of mercury exposure may be due — at least in part — to the slowing of mitochondria. If mitochondria do exhibit a slower velocity after exposure to mercury, the reduced velocity may be due to the initiation of apoptosis due to Cyt c release and/or a reduction or discontinuation of ATP production (Araragi et al., 2003; Passow and Rothstein, 1960). Accordingly, this experiment examined the hypothesis that perturbation of *G. gallus* glial cells with methylmercuric chloride (MeHgCl) will have a hindering effect upon the velocity of mitochondria within these cells. In order to test this hypothesis, we treated cells from the peripheral nervous system of *G. gallus* with an MeHgCl solution and compared the velocity of mitochondria in these glial cells to the velocity of mitochondria in glial cells that were not treated with MeHgCl.

**Materials and Methods:**

*Dissection and Cell Culture*

Dorsal root ganglia and sympathetic nerve chains were harvested from *G. gallus* embryos following the “Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection” protocol (Morris, 2015a). Following the same protocol, harvested cells were dissociated with trypsin and placed into F+ growth medium on coverslips that had been cleaned and treated with both poly-lysine and laminin (Morris, 2015a). The cultured cells were prepared in advance and were stored in an incubator at 37.0°C for later use.

*Control Preparation*

In order to attribute the results to the presence or lack of MeHgCl, a control was necessary for this experiment. The control received treatment that was identical to the experimental condition with the exclusion of MeHgCl. A 40 nM solution of hydrochloric acid (HCl) was prepared by dilution with Hank’s Balanced Salt Solution (HBSS). A petri dish containing one coverslip of cultured cells was obtained from the incubator. The growth medium was removed and set aside. The cells were then submerged into 1 ml of 40 nM HCl in HBSS and were placed into the incubator at 37.0°C for 15 minutes. After removing the cells from the incubator, the cells were washed by discarding the HCl in HBSS, replacing it with enough HBSS to cover the cells, and placing the cells back into the incubator for 5 minutes. The wash step was repeated twice more before the cells were prepared for staining.

Throughout the staining procedure, the stain and treated cells were kept out of the light as often as possible through the use of aluminum foil to avoid bleaching. A 100 nM solution of the lipophilic cationic fluorescent dye, MitoTracker Orange CMTMRos, was prepared by dilution with dimethyl sulfoxide (DMSO). The cells that had been treated with 40 nM HCl in HBSS were removed from the incubator and the HBSS remaining from the previous wash was removed. The cells were submerged in 1 ml of 100 nM MitoTracker Orange CMTMRos in DMSO and were placed into the incubator at 37.0°C for 15 minutes. After removing the cells from the incubator, the cells were washed by discarding the MitoTracker Orange CMTMRos in DMSO, replacing it
with enough HBSS to cover the cells, and placing the cells back into the incubator for 5 minutes. The wash step was repeated twice more. After the final wash, the HBSS was replaced with the growth medium that was set aside at the beginning of the control preparation.

**Experimental Preparation**

The protocol and reagents used in the experimental preparation were nearly identical to those used in the control preparation. The only difference in the experimental preparation was the use of a 40 nM solution of MeHgCl in HCl diluted with HBSS instead of a 40 nM solution of HCl diluted with HBSS.

**Slide Preparation**

Immediately after preparing the control and experimental coverslips, two slides were labeled with their condition (control or experimental) and the experimenter’s initials. In accordance with part B of the “Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells” protocol, separate observation chambers were then created for the control and experimental conditions using the corresponding coverslip and slide (Morris, 2015b).

**Microscopy and Imaging**

In order to avoid cell death due to a lack of oxygen, observation and imaging of the control and experimental slides took place promptly after slide preparation. Both the control and experimental slides were viewed through the use of a Nikon Eclipse E200 microscope. All images were taken using a Spot Insight camera (model 18.2 Color Mosaic) attached to the microscope via a 1.0X Diagnostic Instruments C-mount and a Nikon downtube. The camera was operated by Spot Microscopy Imaging Software (version 5.2) downloaded on an Apple iMac running OS X Yosemite (version 10.10.4).

Prior to imaging, the microscope was aligned for Koehler illumination. Throughout imaging, the temperature surrounding the slide was maintained between 31 and 38°C using a portable heater and a thermometer. Using bright-field phase microscopy, one glial cell was located on the control slide and an image of this glial cell was taken using the 40X objective lens. Subsequently, fluorescence phase microscopy was used to take an image of this glial cell every ten seconds over a period of 90 seconds. The first image was taken at 0 seconds, resulting in a total of ten time-lapse images per glial cell. All fluorescent images were taken with the 40X objective lens and an exposure time of 1500 milliseconds. Importantly, the images taken using fluorescence phase microscopy were saved with file names denoting the order in which they were taken. In total, three glial cells were located on the control slide and three were located on the experimental slide. Each glial cell was imaged according to this protocol. These images were saved for later analysis.
Image Analysis

Fluorescent images of glial cells on the control and experimental slides were analyzed with ImageJ (version 1.49) and all data were recorded and analyzed using Microsoft Excel (version 15.13.1). Both ImageJ and Microsoft Excel were downloaded on an Apple MacBook Pro running OS X El Capitan (version 10.11.1). All time-lapse images taken of the first glial cell on the control slide were opened in ImageJ. One isolated mitochondrion was located in the first image. Each subsequent image was then examined to ensure that this mitochondrion could be distinguished from others in each image. Otherwise, a different mitochondrion was chosen. The selected mitochondrion was enlarged to a value of 3200%. The pixel coordinates of one edge of this mitochondrion were recorded. The same mitochondrion was located in subsequent images, was enlarged to a value 3200%, and the new pixel coordinates of the same edge were recorded (i.e. one particular edge of the mitochondrion was used to track its movement). Using the line tool, a line was drawn from the pixel coordinates recorded from the first image to those recorded from the second image; this line provided a distance (in pixels) between these points, which was recorded in Excel. The distance travelled by this mitochondrion between subsequent images was determined through the same process. In total, three mitochondria were observed in each of the three control and three experimental glial cells. The distance travelled by each mitochondrion between each image was found according to this protocol. Aggregates of 78 and 81 distance values were determined for the control and experimental conditions, respectively.

All distance values were converted from pixels to nanometers (nm) using the “Quick ImageJ Tutorial: Scalebar calibration” protocol; scale bars were created using the same protocol (“Quick ImageJ,” 2009). Velocities were calculated by dividing all distances (in nm) by the time that elapsed between each image, which was 10 seconds. An average mitochondrial velocity and standard deviation were calculated for both the control and experimental conditions. The standard deviations for the control and experimental conditions were then pooled and a t-test was performed with a 95% confidence level to determine whether or not there was a significant difference between the control and experimental mean velocities.
Results:

**Figure 1** – Transmitted light image of glial cell shown in Figure 2. The rectangle surrounds the region that is enlarged and displayed in Figures 2A and 2B. Notice the similarity in the shape of the enlarged region of this glial cell as compared to the shape created by the fluorescing mitochondria in Figure 2.
Figure 2 – Enlarged time-lapse images of stained mitochondria within a glial cell subjected to control conditions (no MeHgCl). Figures 2A and 2B depict the same glial cell and its mitochondria exposed to fluorescent light at 0 and 90 seconds, respectively, during the time-lapse period. The arrows indicate one of the mitochondria from which velocity data were obtained. In Figure 2B, notice the gap that formed between the mitochondrion and the white line over the 90-second period, which is indicative of the mitochondrion’s movement. Additionally, note the change in stain brightness that occurs over said period.
Figure 3 – Transmitted light image of glial cell shown in Figure 4. The rectangle surrounds the region that is enlarged and displayed in Figures 4A and 4B. Notice the similarity in the shape of the enlarged region of this glial cell as compared to the shape created by the fluorescing mitochondria in Figure 4.
Figure 4A – Enlarged time-lapse images of stained mitochondria within a glial cell subjected to experimental conditions (MeHgCl). Figures 4A and 4B depict the same glial cell and its mitochondria exposed to fluorescent light at 0 and 90 second, respectively, during the time-lapse period. The arrows indicate one of the mitochondria from which velocity data were obtained. Notice how similar these images are to the control in terms of mitochondrial movement and stain brightness over the time-lapse period of 90 seconds.
Figure 5 – Average velocity of mitochondria in glial cells subjected to the control (no MeHgCl) and experimental (MeHgCl) conditions (n=78 velocities for the control and n=81 velocities for the experimental). Each error bar illustrates the standard deviation of the average velocity for the corresponding condition. It is important to notice that while the experimental average mitochondrial velocity is lower than that of the control, the values are not far from one another.

Standard deviations of 15.6 and 15.4 nm/s were calculated for the control and experimental conditions, respectively. These standard deviations were pooled, which resulted in a value of 15.5 nm/s. In conducting a t-test, null and alternative hypotheses were formulated. The null hypothesis stated that the average mitochondrial velocities of the control and experimental conditions were the same, whereas the alternative hypothesis stated that the average mitochondrial velocities were significantly different. The experimental t-value was calculated to be 1.70. The tabulated t-value at a 95% confidence level was found to be 1.9752 (“Student’s T,” n.d.). Comparison of the experimental and tabulated t-values resulted in a failure to reject the null hypothesis.
Discussion and Conclusions:

The hypothesis that the perturbation of *G. gallus* glial cells with MeHgCl will have a hindering effect upon the velocity of mitochondria within the affected cells is not supported by the data. The mitochondria in cells treated with MeHgCl exhibited a lower average velocity than the mitochondria in cells treated with control conditions, which appears to support the hypothesis. However, the t-test that was conducted failed to reject the null hypothesis, resulting in the conclusion that there is no statistically significant difference between the average mitochondrial velocities of the control and experimental conditions.

If the experiment had been repeated such that similar trends in results were observed with a statistically significant difference between the average mitochondrial velocities of the control and experimental conditions, then the hypothesis would have been supported. As noted in the introduction, Araragi et al. stated that the presence of various mercury-containing compounds prompted apoptosis in human and mouse cells via the release of Cyt c from the mitochondria (2003). It seems possible that apoptosis due to the release of Cyt c from the mitochondria, or the inhibition of cellular respiration may be accompanied by a decrease in mitochondrial velocity. If Cyt c is released from the mitochondria and prompts apoptosis or if cellular respiration is inhibited when mercury-containing compounds are introduced to *G. gallus* cells, then in the case of a supported hypothesis, these phenomena provide potential explanations as to why the average mitochondrial velocity was found to be lower in glial cells perturbed with MeHgCl than in those treated as a control.

There are a number of means through which this experiment could be refined to increase both the accuracy and precision of the results. Increasing the period of time over which the time-lapse images are taken and the amount of time that elapses between each image would make the mitochondrial movements more distinct (i.e. easier to identify) when analyzing the images. Reducing the exposure time would lessen the extent of mitochondrial stain bleaching similar to that exhibited in Figures 2B and 4B. Lessening the amount of bleaching would make mitochondria and their movements more distinct, and it would increase the number of mitochondria that can be identified in every time-lapse image. Most importantly, running the experiment more than once with new cells would help to confirm or disaffirm that nothing unusual happened during any experimental run.

In addition to refining the experiment to better the results, there are a number of ways to adjust the protocol in order to extend the results in new directions. The experiment could be carried out using a different model organism. It would be especially interesting — but may bring about ethical concerns — to use an organism that has a greater genetic similarity to humans, which may provide some further insight into how mitochondria in human glial cells are affected by mercury-containing compounds. Furthermore, the experiment could be run with increasing concentrations of MeHgCl in order to determine the concentration that is lethal to *G. gallus* glial cells. After determining the lethal dose, *G. gallus* glial cells could be stained and then subjected to this dose through the use of a flow chamber (Morris, 2015c). The mitochondrial velocity within these cells could then be examined for any interesting phenomena.
References:


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

Brandon S. Williams