Effects of methylmercuric chloride on the charge of mitochondria in glial cells of *Gallus gallus*

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Introduction:

Glial cells accompany neurons to compose the nervous system and are vastly more abundant than neurons (Kandel et al., 2013). The energy source required for metabolism and protein synthesis to occur in glia and all cells is adenosine triphosphate (ATP) which is produced by the process of oxidative phosphorylation in mitochondria. Structurally, mitochondria have an outer membrane as well as an inner membrane that surrounds a matrix (Frey and Mannella, 2000). The process of oxidative phosphorylation causes mitochondria to have a negative inner membrane charge (Michelakis, 2008). During oxidative phosphorylation, electrons from NADH or $FADH_2$ in the Krebs cycle are carried by proteins that form the electron transport chain to oxygen in the mitochondrial matrix of the inner membrane of the mitochondria, forming water. As the electrons are being carried, protons from the protein complexes of the electron transport chain are pumped out of the mitochondrial matrix and into the intermembrane space. ATP is then formed once the protons flow back into the mitochondrial matrix and ADP and phosphate are bound. It is the pumping of the protons out of the mitochondrial matrix that creates a proton gradient and results in the organelle having a negative inner membrane charge during this process (Michelakis, 2008). Chemical insults to the cell can alter this function of the mitochondria.

Mercury is an example of a neurotoxic chemical that can greatly affect the function of nervous system cells. According to the United States Environmental Protection Agency, most people have a small amount of mercury within them, indicating that mercury is a toxin that is widely present in the environment (*U.S. Environmental Protection Agency*). The effects of mercury exposure are not usually seen at this level (*U.S. Environmental Protection Agency*). However, higher dose exposures, mainly due to the consumption of fish containing high levels of mercury, or from using products that contain mercury, can have drastic neurologic effects, including motor and cognitive changes and even death (*U.S. Environmental Protection Agency*). According to a review on proposed mechanisms for neurotoxicity by mercury, mercury may target microtubules, neurotransmitter production and release, and DNA, RNA and protein synthesis, among other cellular functions, leading to these neurological effects with mercury poisoning (Castoldi et al., 2001). A different study by Pamplett et al found that the mitochondria within the motor neuros of mice who were exposed to mercury vapor were swollen, which indicated to these researchers that mercury caused damage to the membranes of mitochondria (Pamphlett et al., 1998).

This experiment will test whether mercury has an effect on mitochondrial function in glia by using MitoTracker® Orange fluorescent dye as a marker for mitochondrial charge. MitoTracker® Orange stains mitochondria based on membrane potential, and has an increased brightness intensity when the mitochondria have a more negative membrane potential (Krysko et al., 2001 and *Thermo Fisher Scientific*). A more negative membrane potential of the mitochondria indicates increased metabolic activity. It is hypothesized that glial cells that are exposed to mercury will have a decreased MitoTracker® Orange fluorescence intensity compared to glial cells that are not exposed to mercury, meaning that the mitochondria of the glial cells that are exposed to mercury will be less metabolically active than the mitochondria of glial cells that are not exposed to mercury. This experiment was conducted using dorsal root ganglia and sympathetic chains of 10 day old chick embryos (*Gallus gallus*). Chick embryos were chosen for this experiment as the development of the nervous system of chick embryos is similar to that of humans, and therefore serve as a model with applications for the human nervous system (Davey and Tickle, 2007). This experiment was conducted in collaboration with Brandon Williams and Guanjia Pan.

Materials and Methods:

Dissection

This experiment was conducted using 10 day old chick embryos (*Gallus gallus*) which were harvested and dissected as per protocol "Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection" (Morris, 2015a). Dorsal root ganglia and sympathetic chains were dissected and collected. Coverslips plated with a mix of ganglia chunks and dissociated cells were used for this experiment.

The ganglia chunks and dissociated cells were grown on coverslips that had been treated per protocol "Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection" (Morris, 2015a). The coverslips were first cleaned with absolute ethanol and then autoclaved for sterility. The coverslips were then treated with poly-lysine for about 30 minutes followed by a rinse with sterile water. Once dry, the coverslips were then treated with laminin for about 30 minutes followed by a rinse with Hank's Balanced Salt Solution (HBSS). The wet, treated cover slip was then placed into a 35mm petri dish that contained F-plus growth medium.

The dissociated cells and ganglia chunks were plated dropwise onto these treated coverslips in F-plus growth medium and were incubated at 37˚C for about 24 hours before further experimental treatment.

Control Preparation

A dilution 1:100 of hydrochloric acid (HCl) in HBSS was applied to the control coverslip, to account for the HCl that was contained in the solution that was applied to the experimental coverslip. To apply the HCl solution, the F-plus growth medium was first removed with a Pasteur pipette and then immediately replaced with approximately 1ml of the HCl solution. The petri dish containing a coverslip of cells and 1ml of HCl solution was incubated at 37˚C for 15 minutes.

After the 15 minute incubation, the HCl solution was removed with a Pasteur pipette. Almost immediately after removal of the HCl solution, 1ml of HBSS was expelled into the petri dish to wash the cells. The petri dish containing a coverslip and 1ml of HBSS was then

incubated for 5 minutes at 37˚C. The cells were washed a total of 3 times with 1ml of HBSS following this procedure, and were incubated for 5 minutes at 37˚C after each removal and addition of 1ml of HBSS.

Experimental Preparation

A solution of methylmercury (MeHg) in HCl diluted 1:100 to 40nM in HBSS was applied to the experimental cells. To apply the MeHgCl solution to the cells, the growth medium was removed and immediately replaced with 1ml of the MeHgCl solution using Pasteur pipettes. The petri dish containing the coverslip of cells and 1ml of MeHgCl was incubated for 15 minutes at 37˚C.

The MeHgCl was then removed and immediately replaced with 1ml of HBSS to wash the cells. The petri dish containing the coverslip of cells and 1ml of HBSS was incubated for 5 minutes at 37˚C. This wash procedure was repeated so that the coverslip of cells underwent 3 washes with 1ml of HBSS.

Fluorescent Dye

MitoTracker® Orange prepared at a concentration of 100nM in dimethyl sulfoxide (DMSO) was the fluorescent dye that was used in this experiment.

Both the control and experimental groups underwent the same fluorescent labeling procedure, which was performed after the previously described control and experimental preparation. After the third wash with 1ml of HBSS and the 5 minute incubation at 37˚C that followed for the control and experimental preparations, the HBSS was removed and was immediately replaced with 1ml of MitoTracker® Orange using Pasteur pipettes. The petri dish containing the coverslip of cells and 1ml of MitoTracker® Orange was incubated for 15 minutes at 37˚C.

The Mitotracker® Orange was then removed and was immediately replaced with 1ml of HBSS to wash the cells. The petri dish containing the coverslip of cells and 1ml of HBSS was incubated for 5 minutes at 37˚C. This was repeated so that the cells were washed a total of 3 times with 1ml of HBSS and were incubated for 5 minutes at 37˚C between each wash. After the third wash and 5 minute incubation period, the HBSS was removed and immediately replaced with fresh F-plus growth medium. The coverslips were then prepared for microscopy, described below.

Microscopy and Imaging

Both the control and experimental coverslips were prepared and placed on slides using the chip chamber technique as per protocol "Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells" (Morris, 2015b). A Nikon Eclipse E80i Epi Fluorescence microscope was used to view and take images of the cells, with an objective lens of 40X PH2. Fluorescent images were taken using light labeled G 2E/C.

Glial cells were imaged individually using a SPOT RTS Diagnostic Instruments camera with a camera mount of 0.76X on an Apple iMac with OS X Yosemite version 10.10.4. The software used to image the cells was SPOT Software 5.2. Glial cells were identified using the brightfield view on the microscope and then one transmitted light image was taken for each glial cell. After capturing a transmitted light image, fluorescent images with the same field of view were taken with an exposure time of 500ms. For the control, 19 glial cells were imaged, and for the experimental slide 16 glial cells were imaged.

Data Analysis

The glial cells that were analyzed for this experiment were glial cells that were single and isolated on the coverslip, meaning that the glial cells were not in contact with other glial cells, nor were they in contact with neurons, axons, nor any part of another cell. The program ImageJ 1.50a was used to analyze the data for the intensity of fluorescence. The transmitted light image was used to compare to the fluorescent image of the same glial cell, to identify and locate the cell. Since the fluorescent dye did not label the entire cell, but rather the mitochondria within that cell, the perimeter and area of the glial cell in the fluorescent image appears lesser than the perimeter and area of the glial cell in the transmitted light image. This decrease in perimeter and area from the transmitted light image to the fluorescent image indicates that the mitochondria are not present in all spaces within the cell, and therefore not all the space of the cell was labeled with the fluorescent dye. Therefore, measuring the perimeter of only the fluorescent area of the cell instead of the entire area of the cell based on the transmitted light image (which would include areas without fluorescence) was determined to be sufficient to collect data for this experiment, since the background or areas of no fluorescence would be subtracted from the mean brightness of the fluorescence anyway, as described later. Areas of fluorescence within glial cells were defined as areas that had mean brightness values at least 1.5 times the brightness of the background.

The free-hand polygon tool was used to trace one cell around the fluorescent perimeter, and ImageJ was set to measure the area of the selection of the polygon tool, the integrated density and the mean gray value. The mean value was a measure of the average fluorescent brightness value for the area selected, which is measured on 256 different levels of gray where 0 is black and 255 is white. This mean fluorescent brightness value was measured and recorded for each glial cell. Three areas of background that did not contain fluorescence were also selected and the mean fluorescent brightness values for the three background areas were recorded for each glial cell. The three background means were then averaged and subtracted from the mean brightness of the selection of the cell to calculate a mean fluorescent brightness for each glial cell.

Results

Figure 1a. Transmitted light image at of a single, isolated control glial cell at 40X PH2 magnification. Control glial cells were treated with a dilution 1:100 of HCl in HBSS. The cell of interest in this image is labeled Cell A. Image was taken in collaboration with Brandon Williams and Guanjia Pan.
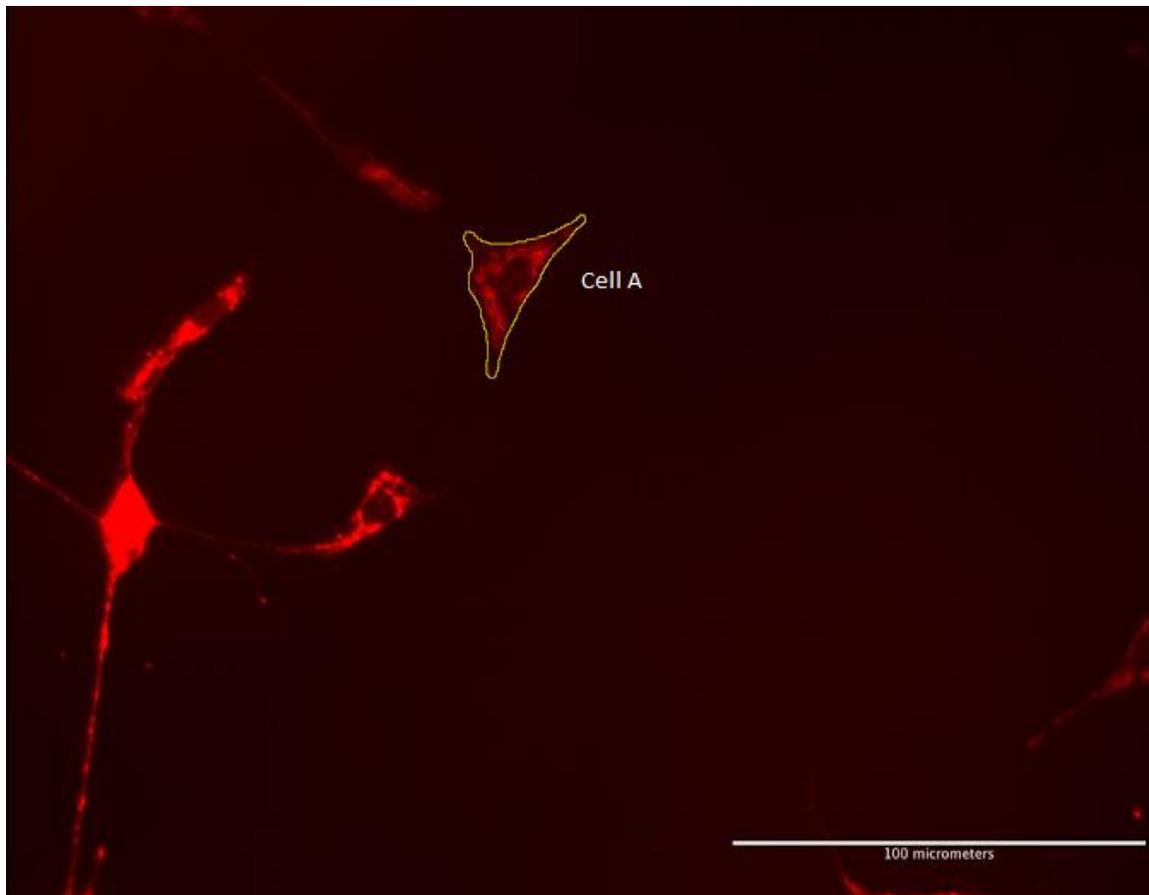
Figure 1b. Fluorescent image of control glial cell at 40X magnification with tracing of fluorescent perimeter in ImageJ. The glial cell of interest is once again labeled Cell A for ease of recognition from the transmitted light image (Fig 1a). In this image, the mitochondria of the glial cell fluoresce based on their membrane potential as a result of being labeled with MitoTracker® Orange. Image was taken in collaboration with Brandon Williams and Guanjia Pan.
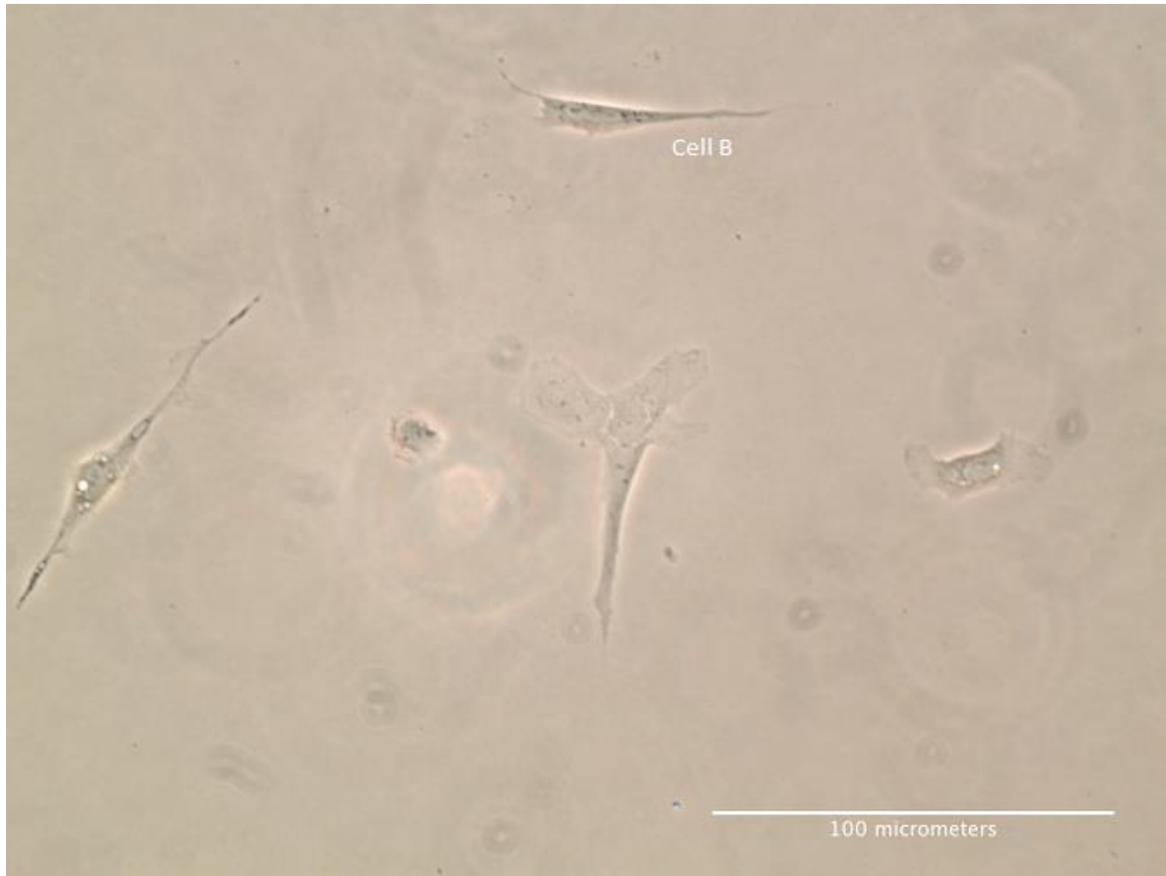
Figure 2a. Transmitted light image of a single, isolated experimental glial cell at 40X PH2 magnification. Experimental glial cells were treated with 40nM MeHgCl in HBSS. The glial cell of interest in this image is labeled as Cell B. Image was taken in collaboration with Brandon Williams and Guanjia Pan.
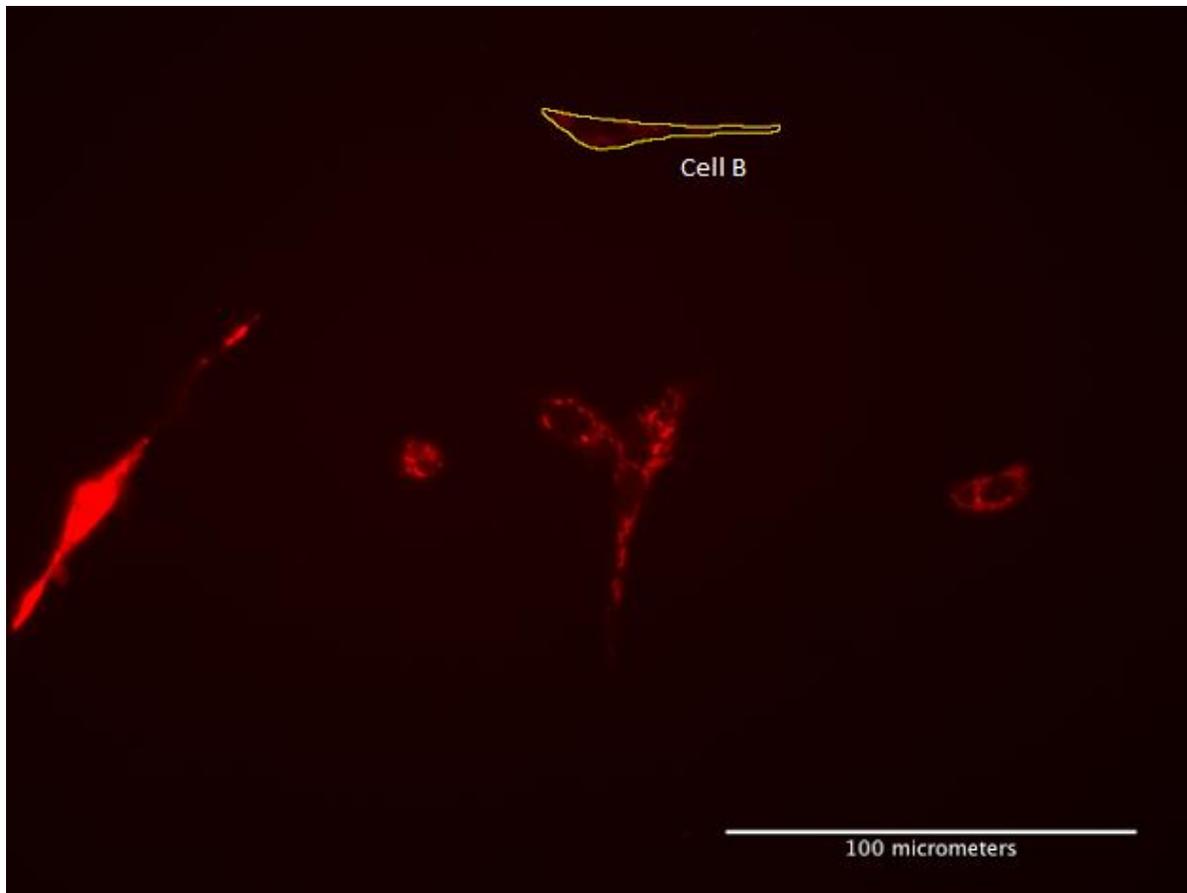
Figure 2b. Fluorescent image of experimental glial cell at 40X magnification with tracing of fluorescent perimeter in ImageJ. The glial cell of interest is once again labeled Cell B for ease of recognition from the transmitted light image (Fig. 2a). In this image, the mitochondria of the glial cell fluoresce based on their membrane potential as a result of being labeled with MitoTracker® Orange. Notice that Cell B in this image is visibly less bright than Cell A in Fig. 1b. Image was taken in collaboration with Brandon Williams and Guanjia Pan.
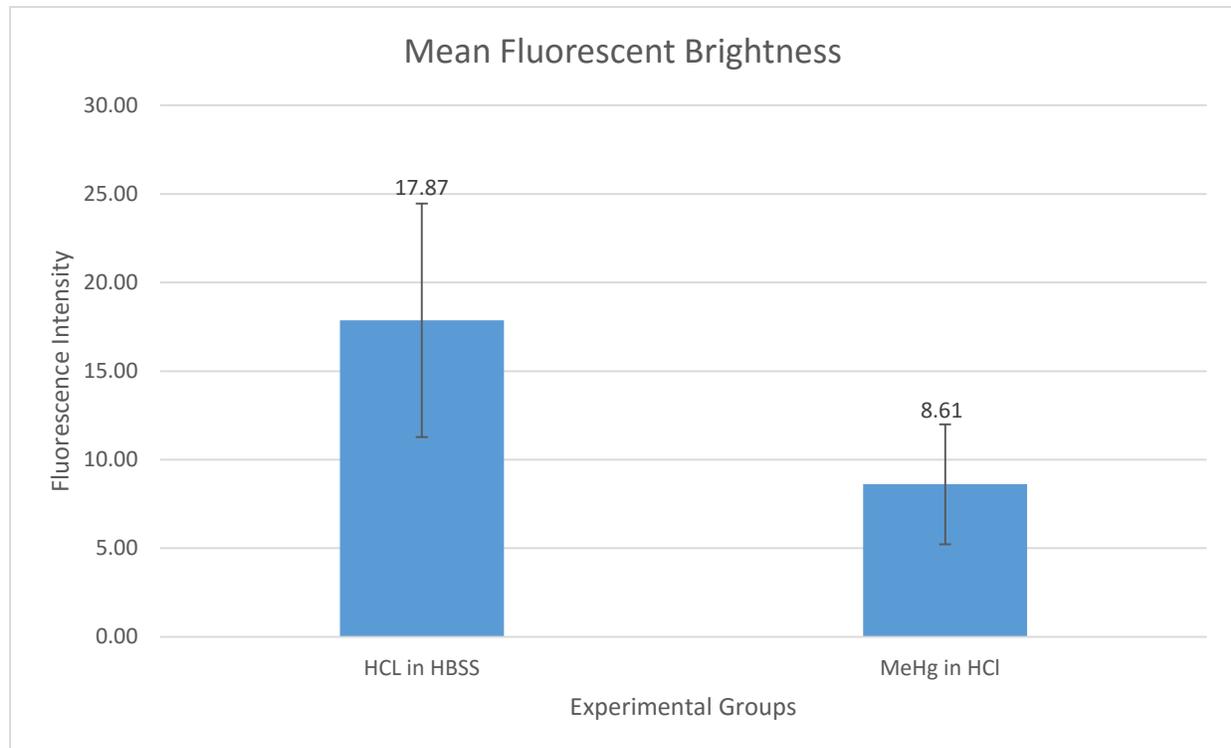
Figure 3. Average mean fluorescence brightness values for each of the experimental groups (n=19 glial cells for the HCL in HBSS group; n=16 glial cells for the MeHgCl group) with standard deviations shown. Notice that the experimental glial cells treated with MeHgCl had a decreased average mean fluorescence brightness value compared to the control glial cells that were treated with HCL in HBSS.

The results show that although there is a difference in the average mean fluorescence brightness values between the control group of glial cells treated with HCl in HBSS, which showed a greater mean fluorescence intensity, and the experimental group of glial cells treated with MeHgCl, which showed a lesser mean fluorescence intensity, the difference in the average mean fluorescent brightness values is not statistically significant (Fig. 3).

Discussion

The hypothesis that the control group of glial cells treated with HCl in HBSS would have a greater mean fluorescence intensity compared to the experimental group of glial cells treated with MeHgCl was partially supported. While the control group of glial cells had a greater mean fluorescence intensity compared to the experimental group, the data was found to not be statistically significant. Perhaps if this experiment was conducted many times or if there were larger sample sizes for the conditional groups, the data would have been statistically significant. The conclusion could then be made that the mitochondria of glial cells that are treated with MeHgCl have a decreased mean fluorescence intensity compared to the mitochondria of a control group of glial cells. Since MitoTracker® Orange labels mitochondria based on having a negative inner membrane potential, and because oxidative phosphorylation leads to this negative

inner membrane charge of mitochondria, it could be concluded, with statistically significant data, that the mitochondria of the glial cells treated with MeHgCl had a reduced rate of oxidative phosphorylation and therefore a reduced production of ATP to be used as energy for the cell.

Various studies have been conducted on the effect of mercury on the nervous system, offering explanations for the mechanisms behind the results of this experiment. One study tested the effects of mercury on granule cells, neurons found in the cerebellum, related to glutamate transport, and found that the release of glutamate increased in cells treated with mercury but that glutamate uptake was inhibited (Fronfría et al., 2005). Additionally, the study found that mitochondrial activity and ATP production were reduced (Fronfría et al, 2005). A different study contradicts the results found in this current study by concluding that there was an increase in the release of creatine, which controls energy homeostasis, when cells were treated with mercury, which prompted gliosis and an increase in metabolic activity in glial cells as a neuroprotective mechanism (Van Vliet et al., 2008). This study found decreased GABA and choline levels of neuronal cells treated with mercury, suggesting that a decreased amount of neurotransmitter release due to the effects of mercury on neurons may stimulate a response from glial cells (Van Vliet et al., 2008). However, perhaps this response is dose-dependent, and perhaps the dose of mercury was too high in the current experiment to promote a neuroprotective response from the glial cells, as the glial cells in this experiment showed decreased metabolic activity. Studies of mitochondria suggest that chemical insults to the cell can trigger oxidative stress in mitochondria to release ROS (reactive oxygen species) which can target the mitochondrial DNA, which encodes the proteins needed for electron transport and oxidative phosphorylation to occur (Ott et al., 2007). Targeting and destroying the DNA needed for these processes to occur would result in decreased and eventually ceased ATP production, and eventually lead to cell death (Ott, et al., 2007). This mechanism of oxidative stress leading to a decrease in metabolic activity could be occurring in the mitochondria of the glial cells of this experiment.

While significant sources of error were minimal, if at all present, possible sources of error in this experiment include the imaging process, as the background visibly contains fluorescence in some of the images. This should have been corrected with the data analysis, as the mean brightness of the background of the image was taken into account, but remains as a possible source of error, especially for images where the background was brighter in some areas than others. Additionally, this experiment was conducted with only one trial of control and experimental cells, so multiple trials with larger sample sizes of glial cells could strengthen the experiment in the future and produce more conclusive data. Other future experiments could include testing a variety of other neurotoxic substances and their effects on glia and mitochondrial activity. This would be especially interesting, as the increase or decrease in metabolic activity in glia could indicate whether or not glia respond in a neuroprotective way to certain neurotoxins. This experiment could also be expanded to test different dosages of mercury or other neurotoxic substances on glia. This could once again have implications on how glia respond to chemical insults to the central nervous system, and the determination of a maximum or lethal dosage that would result in cell death of the glial cells rather than a neuroprotective response.

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

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