Analysis of Mitochondria within Embryonic Peripheral Neurons: The Effects of Methylmercury on Mitochondrial Movement

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Introduction

Mitochondria are double membrane organelles located within the cytoplasm of mammalian cells. This organelle is commonly referred to as the "powerhouse" of the cell because it produces the majority of energy for survival. Mitochondria contain their own genome responsible for encoding the subunits of the respiratory chain that enables the flow of energy through the organelle (Picard & McEwen, 2014). With this flow of energy, mitochondria are able to produce ATP and therefore support functions like ion transport and neurotransmitter biosynthesis to name only two (Picard & McEwen, 2014). Previous studies have also indicated that this organelle is directly influenced by extracellular signals and adapts to changes in the metabolic environment (Picard & McEwen, 2014). Of great sensitivity to mutations of the mitochondria is the brain, therefore indicating that this organelle plays a crucial role in regulating neural functioning (Picard & McEwen, 2014).

Within neurons, mitochondria are located in areas that require a great amount of energy such that they are not located uniformly within the cell (Verburg and Hollenbeck, 2008). The mitochondria are also known to actively move about the neuron traveling from the soma, which is the cell body, to the axon and back based on where the most energy is needed and at what time (Picard & McEwen, 2014). Because of this, mitochondrial movement is of importance to this study. Energy production is key to the survival of a cell, which includes being able to function in the manner for which the cell was designed to do so. The objective of this study was to analyze this movement and determine if it was altered when exposed to the toxic substance methylmercury.

Methylmercury is the organic form of mercury that is already a known environmental pollutant (Costas-Mora, Romero, Lavilla, & Bendicho, 2014). Methylmercury though is more harmful to living systems than inorganic species because it has the ability to cross biological membranes and it is lipid soluble (Costas-Mora, Romero, Lavilla, & Bendicho, 2014). Methylmercury is formed from the methylation of inorganic mercury which is commonly released

into the environment due to the burning of fossil fuels. Human exposure is readily in the form of fish consumption, and poisoning can occur due to the amplification methylmercury undergoes as it progresses through the food chain. Methylmercury has also been identified as having potent neurotoxicity and can perpetuate damage to the brain (Costas-Mora, Romero, Lavilla, & Bendicho, 2014). It is this factor that is of interest and provides the compelling argument that such a substance should be studied. The exact mechanism of how methylmercury causes central nervous system damage is unknown. The fact that neuronal function depends on mitochondrial energy production though, can provide a basis for determining how methylmercury causes such harm.

Therefore, chick embryonic peripheral neurons dissected from the *Gallus gallus* were utilized for this experiment. Embryonic neurons were used in this study because at that stage the cells are constantly dividing having the ability to provide an ample amount of neuronal growth. Mitochondrial movement was viewed under control conditions as well as under the condition of acute methylmercury exposure. Fluorescent microscopy was used to determine the location of the mitochondria and measurements of the distance between selected mitochondria were taken to determine if any movement was occurring. This protocol was completed to study the hypothesis, if a neuron is exposed to an acute dosage of methyl mercury, then the location of mitochondria within the neuron will change due to movement. The testing of this hypothesis is crucial because movement of mitochondria is dependent upon energy need, if movement is occurring due to exposure to methyl mercury than it is possible that areas in need of energy production are not receiving such activity.

Materials and Methods

Embryo Explanting

Following the protocol, *Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION*, 9-day-old chick embryos were dissected for dorsal root ganglia and sympathetic nerve chains (Morris, 2014). Minor changes though were made to the procedure to ensure optimal neuronal growth, more specifically, axonal growth. Instead of treating the coverslips with poly-lysine for roughly 20 minutes, the coverslips were treated for 3 hours with poly-lysine. Following that, treatment with laminin was increased to 2 hours instead of 20 minutes as well. The increased treatment time of both these substances was completed to increase adhesiveness and signaling, respectively, of neuron growth upon the coverslip.

Other changes to the primary tissue culture procedure included increasing the concentrations of nerve growth factor and glutamine within the growth medium. The increased amount of nerve growth factor within the medium

provided even more signaling for the growth of neurons, and the increased amount of glutamine allowed for prolonged presence within the medium because otherwise it easily breaks down. The concentration of nerve growth factor was increased to 200ng/ml from 50ng/ml and the concentration of glutamine was increased to 4mM from 2mM.

To further ensure increased axonal growth, changes were made to the original procedure that included moving the tray forward in the incubator to decrease shelf vibrations, plating 60 ganglia and 6 sympathetic chains instead of 40 and 4 respectively, and dissecting the embryos a day earlier than scheduled to provide 38 hours of growth time versus 24 hours.

Fluorescent Staining

After the embryonic plating was completed, the neurons within the culture were treated with an 118nm solution of Mitotracker to produce a fluorescent stain following the Vital-stain Fluorescence Microscopy procedure within the *Primary Culture of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION of LIVE CELLS* (Morris, 2014). Minor changes were made to this procedure including an increased incubation after the addition of the Mitotracker to 15 minutes, and instead of being diluted in growth medium, the Mitotracker was diluted in DMEM. A flow chamber was made for the control condition as well as for the experimental condition following the protocol Make Flow Chamber from Unstained Coverslip also within *Primary Culture of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION of LIVE CELLS* (Morris, 2014).

Data Collection and Analysis

Data collection consisted of gathering images of the newly grown neurons over the 38-hour incubation period and determining at what positions the mitochondria were located. Images were first taken of the control condition in which no methyl mercury was added. A Nikon 3200 Eclipse fluorescent microscope was used along with a 40X objective lens. The connected camera utilized was a Spot Insight Fire Wire 2 megasample. After achieving Kohler Illumination, neurons with very apparent axons were brought into focus and snapshots were taken first without the addition of fluorescent light utilizing the Spot computer software. Completion of these photos led to snapshots taken with fluorescent light. Multiple neurons were imaged per single coverslip.

In order to mimic the experimental conditions, flow through with DMEM was completed after the first set of snapshots was taken as mentioned above. After the flow through, pictures before and after fluorescent exposure were taken for the same neurons that were observed above. Collection of these images summed up the control data and the experimental set was then imaged. In the case of this study, the experimental condition is a flow through using a 4nM solution of methyl mercury. This is considered to be a low dosage. Images were collected using the same procedure as

the control image collection except that instead of completing the flow through with DMEM, the 4nM solution of methyl mercury was used.

These images were then compared to determine differences in mitochondrial location between methyl mercury exposure and non-methyl mercury exposure. Data analysis was completed using the software ImageJ to measure the distances between selected mitochondria. Ten mitochondria were selected for analysis and selection was based on how bright the mitochondria were and how isolated. To measure the movement of mitochondria, both the control images and the experimental images were opened in the ImageJ software. Following that, the measure feature of the software was utilized. The measurement was set to measure length and determining the amount of pixels within a millimeter and then converting that to the amount of pixels within a micrometer set the scale. Utilizing an image of a millimeter under the same microscope conditions mentioned above, the amount of pixels for that length was calculated using the ImageJ software.

Once the scale was set, distances between chosen mitochondria were measured. Using the measurement tool, lengths were collected from one mitochondrion to another for both the control images and the experimental images. Utilization of the images taken before fluorescent exposure determined which mitochondria would be measured and then the same were looked at in both studied conditions in order to compare if any distances between the mitochondria were different. If a difference in length was measured, then mitochondrial movement had occurred. All distances were collected for the same ten mitochondria in each condition and a graph was made to compare the results using Microsoft Excel.

Results

After the 38-hour growth period, images of neurons were collected. Neuron growth and axonal anchorage were dense because of the above measures taken. Populated areas were sought after for imaging so as to provide a snapshot of general mitochondrial location. Figure 1 represents an example of the type of images that were used for analysis. It is also an image before exposure to fluorescent light to provide a reference for determining location of axons and mitochondria within the fluorescent images.

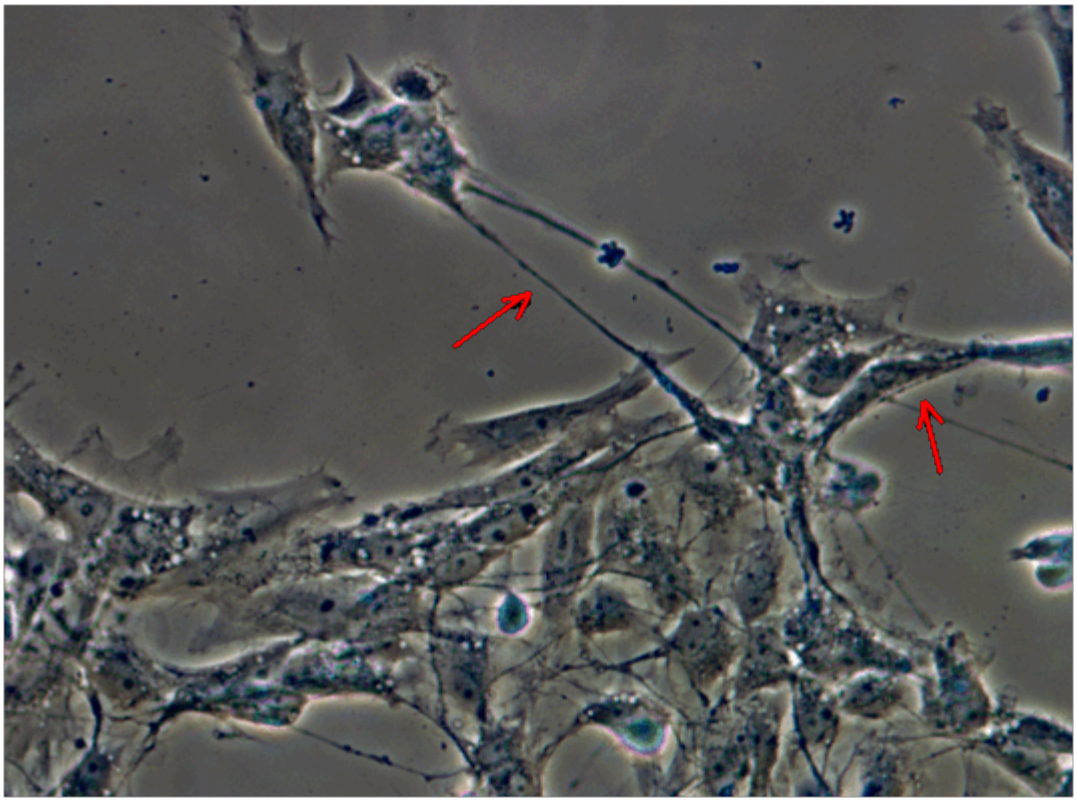


Figure 1: Neuronal growth before fluorescent exposure. Taken using Spot software, this image represents axonal growth and potential mitochondrial positioning for reference when analyzing under fluorescent exposure. Prominent areas are indicated. (n=10).

Under fluorescent exposure and due to the addition of the mitotracker, the mitochondria were illuminated and location therefore was detected. Due to the density of the neurons as well as the glial cells, only areas of isolated mitochondria were utilized for measurement collection. Figure 2 is an image of the control condition before the addition of methylmercury.

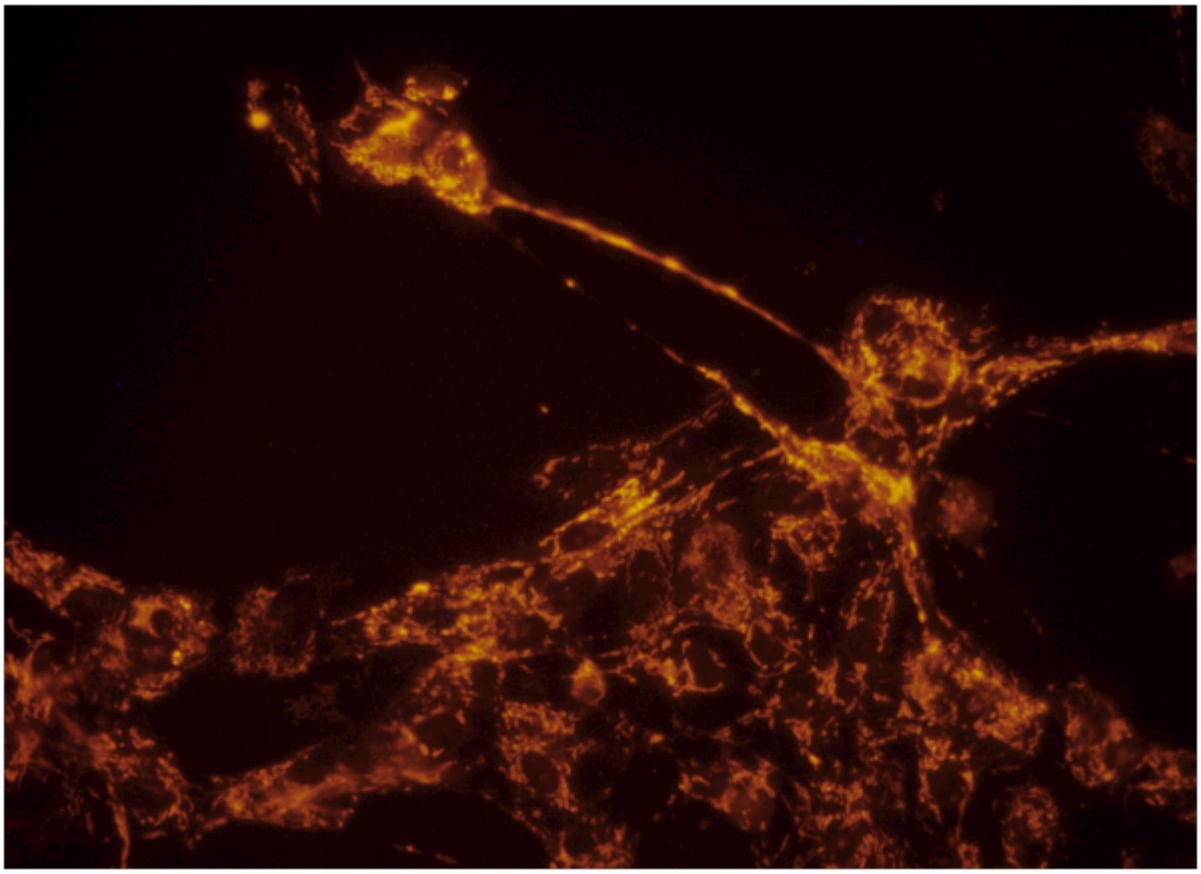
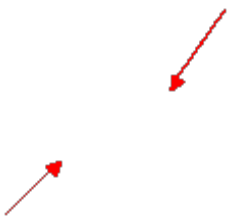


Figure 2: Neuronal growth after fluorescent exposure. Taken using spot software, this image represents the mitochondria position before methyl mercury exposure. Examples of the chosen mitochondrion for analysis are indicated. (n=10).

Viewing again under fluorescent exposure, images were taken of the neurons after methylmercury exposure. The mitotracker illuminated the mitochondria in this condition as well and the same mitochondria that were pinpointed in the control condition were also pinpointed in the experimental condition.



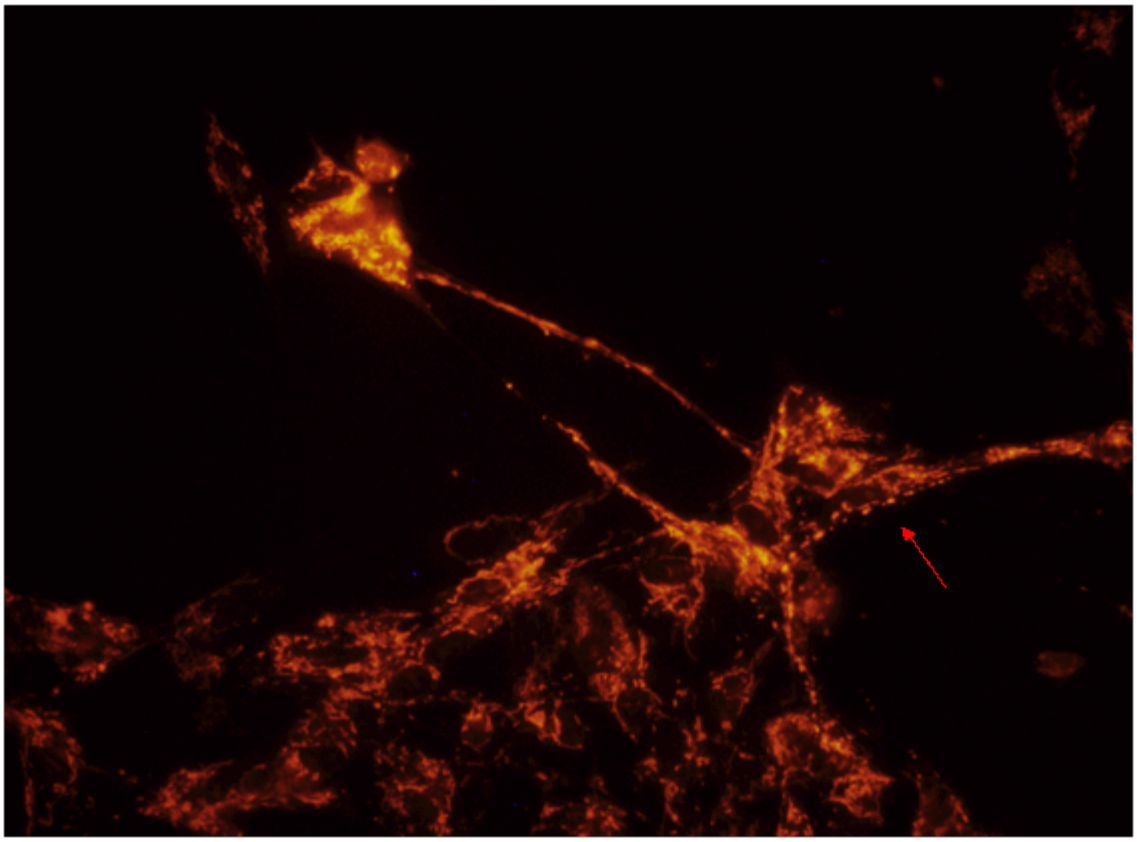


Figure 3: Neuronal growth after fluorescent exposure. Taken with Spot, this image represents the mitochondria position after methyl mercury exposure. Examples of the chosen mitochondrion for analysis are indicated. (n=10).

After compilation of mitochondria locations, in order to determine movement the same distances between mitochondria were measured for both the control and experimental results. Of the brightest, most isolated mitochondria, distances between the mitochondria appeared to decrease after methylmercurey exposure. The average decrease in distance was 202μm. Figure 4 represents the ten different distances measured in both the control and experimental condition.

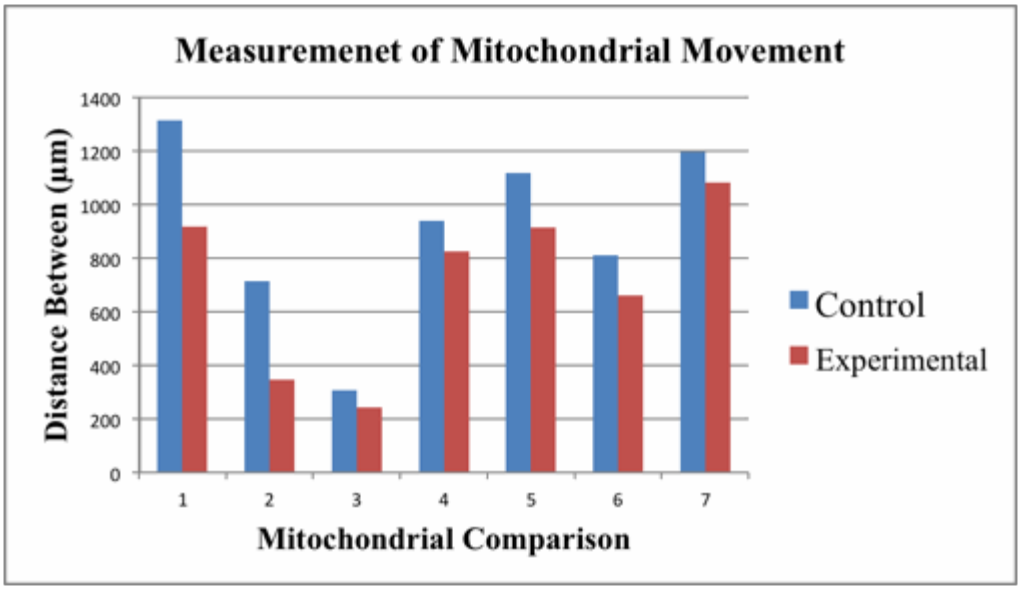


Figure 4: Measurement of mitochondrial movement. Created using Excel, this graph represents the measured distances between the same ten mitochondria for both the control condition and the experimental condition. (n=10).

Conclusions

Due to the decreased distance between mitochondria after exposure to methylmercury, it can be concluded that the hypothesis of mitochondrial movement and differing mitochondrial location after exposure can be supported. The evidence suggests that mitochondria are responding the methylmercury exposure by moving locations within the neuron and more specifically within the axon. Although the n value is lower, mitochondria chosen specifically for brightness and isolation rules out any bias and provides a representative picture of behavior that could potentially be applied to the large scale behavior. With an increased n value and results imitating the ones presented in this study, the conclusion could be drawn that strong support is present for methylmercury exposure influencing movement of mitochondria within neurons.

The results of this study show only that it is possible for methylmercury exposure to induce mitochondrial movement, not how such movement will influence cell function. It may be that the movement will disrupt energy production due to the nature of mitochondria and the purpose of their location. This remains unknown though and is a factor that can and should be studied in the future. If ample amount of time was allotted, this study would tremendously benefit from a larger n value, several repeats to rule out any human error, and less dense images to view individual mitochondrial movements. This last point is one in which would greatly improve the study. To be done differently if repeated, images of single neurons would be utilized so that not only mitochondrial movement could be looked at, but also the direction in which the mitochondria are moving to after exposure. Also as another refinement of error,

measurements of distance between mitochondria should be supplemented with distances from actual cell bodies, growth cones, or nuclei. This will provide a sense of direction to the movement, which can contribute to the behavior this movement, is creating. As a final point on improving this experiment when repeated, on observation chamber could be utilized instead of a flow chamber to ensure that axons are not being dislodged due to the fluid movement.

Experiments to follow this study should be ones that test behavior of cells due to movement and ones that test the effects of different dosages of methylmercury. Testing behavior influenced by mitochondrial movement will allow for the connection to be made between location, energy need, and cell function under the influences of methyl mercury. A test can be done to determine if the mitochondrial movement results in apoptosis after exposure the methylmercury as an example. Studying different doses of methlymercury will allow for the discovery of dose-dependent results if any exist. The question can be asked, does a higher dose affect the intensity of mitochondrial movement? Due to the known information that energy production is required for cellular function and that within neurons location of mitochondria indicate areas of high energy needs (Picard & McEwen, 2014), it is crucial to continue these efforts of deciphering the exact mechanism of methylmercury and its toxicity to the brain.

References

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