Methyl mercury induced neurotoxicity on mitochondria in embryonic neurites

Moya Willis

Neurobiology Short Report

Bio324 / Neurobiology

Wheaton College, Norton, Massachusetts, USA

April 23, 2014

Introduction

Neuronal cell have a high number of regions where ATP is essential for function (Verburg, 2008). Neurons depend on mitochondria trafficking for survival. They play a key role in normal cell functioning by producing energy in the form of ATP. The electron transport chain releases energy that is used to pump H+ against their concentration gradient from the matrix of the mitochondrion and into the intermembrane space (Mannella, 2013). The active transport of protons into the intermembrane space creates a proton gradient. The protons are transported back to the mitochondrial matrix through ATP synthase within the mitochondrial membrane. ATP is generated from the energy of the protons and ADP.

The maintenance of mitochondrial membrane potential is vital for ATP synthesis. The membrane potential of the mitochondria is negative due to the proton gradient created on the intermembrane space. The gradient is essential for calcium uptake, which is used to maintain calcium homeostasis in the cell (Verburg, & Hollenbeck 2008). Mitochondrial depolarization is usually transient. A permanent change in mitochondrial charge by a toxin indicates the onset of cell death (Griffiths, 2000). The mitochondria respond to environmental changes in the axon as demonstrated by the anterograde motor direction during growth and retrograde motor direction when blockage is present (Morris and Hollenbeck, 1993). The vulnerability of the neurons is age dependent. In this experiment embryonic cells are used

because the developing nervous system has been shown to be less efficient at recovery from MeHg exposure. A previous study using rats of various ages have found that, MeHg increases the level of reactive species of oxygen in the brain and alters the intracellular calcium levels more for younger rats (Dreiem & Seegal, 2007). Another experiment using rat striatal synaptosomes shows that exposure to methyl mercury reduced mitochondrial metabolic function and mitochondrial membrane potential (Dreiem, 2005).

The neurotoxic effects of MeHg are well studied. However, the mechanism which brain induces damage is less studied. One proposed mechanism is that MeHg triggers a mechanism in the mitochondria that increases calcium levels in the cytosol resulting in reduced mitochondrial function. Additionally, mitochondria organelles are the primary site for production of reactive oxygen species. The accumulation of MeHg in the cell changes mitochondria enzyme functions and decreases the respiratory process of using reactive oxygen to produce ATP. Additionally, there is an increase in the efflux of Ca^{+2} and a decrease in mitochondrial charge. The increase in intracellular calcium causes mitochondrial voltage to become less negative and induces the toxic effects on the CNS (Dreiem, 2005). We hypothesize if embryonic neurons are exposed to methylmercury, then there will be a reduction in overall voltage in the axonal mitochondria. To test this hypothesis, we exposed embryonic neurons to methyl mercury and measured the brightness of axons as direct consequence of axon voltage and mitochondria functioning

Materials and Methods

Primary Cultures

Primary cultures were obtained from dissecting day 10 *Gallus gallus* embryos for sympathetic nerve chains and dorsal root ganglia. The techniques used to isolate the cells can be found in “Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection” (Morris, 2014a). The DRGs and sympathetic chains were dissociated using 0.1% trypsin solution. After incubation period of 20 minutes, cells were placed on coverslips treated with lamin and poly-L-lysine. The methods used to treat the coverslips are outlined in “Primary Culture of Chick Embryonic Peripheral

Neurons 2: Observation of Unlabeled Cells" (Morris, 2014b). Change to the procedure includes 3 hours of poly-L-lysine treatment to increase cell adhesion to the coverslips. A total of 60 ganglia and 6 sympathetic chains were plated to increase clusters of neurons for imaging. The dishes were isolated on a storage shelf to reduce disturbance from vibration of incubator for better anchoring of the cells onto coverslips. The NGF was increased to 200 ng/mL and 4nM of glutamine was added. These changes increased growth signals and allowed quicker breakdown. The cell cultures were suspended in Dulbecco's modified Eagle medium (DMEM) instead of HBSS and incubated at 37 degrees Celsius for 38 hours to optimize growth of the cells.

The control group had no addition of methylmercury (- CH3Hg+) in DMEM, while the experimental groups had an addition on methylmercury (+ CH3Hg+) in 40nM DMEM. DMEM was pipetted off the cells after 38-hour incubation. A DMEM solution of either -/+ CH3Hg+ was added. The cells were incubated for 20 minutes. Then the growth medium and -/+ CH3Hg+ was removed from dishes. Mitotracker was added to stain the axon mitochondria then sample incubated for 10 minutes. The stain was replaced with DMEM.

Flow Chamber

The live cells were observed in a flow chamber to allow manipulation in the control and experimental dish. Images were captured with Nikon Eclipse E200 microscope with phase contrast and fluorescent at magnification 40x for control and experimental group. A full description for creating the flow chamber is described in "Primary Culture of Chick Embryonic Peripheral Neurons 3: Staining and Observation of Live Cells" (Morris, 2014c).

Statistical Analysis

The brightness of axons is analyzed using mean ± standard deviation. Results were collected from one trial using one treatment and control group. The images were captured using Sony DFW-x700 with a 1.0X C-mount camera processed by BTv software. The exposure times of the images were 525.5ms in fluorescent lighting. The images were exported to ImageJ software and converted to 8-bit grayscale with display range of 0-255 pixels. The images were

normalized for brightness. To normalize the images, the brightness of both images was adjusted to the ratio value for both images. The following formula was used to calculate the ratio between the brighter image compared to the dimmer image

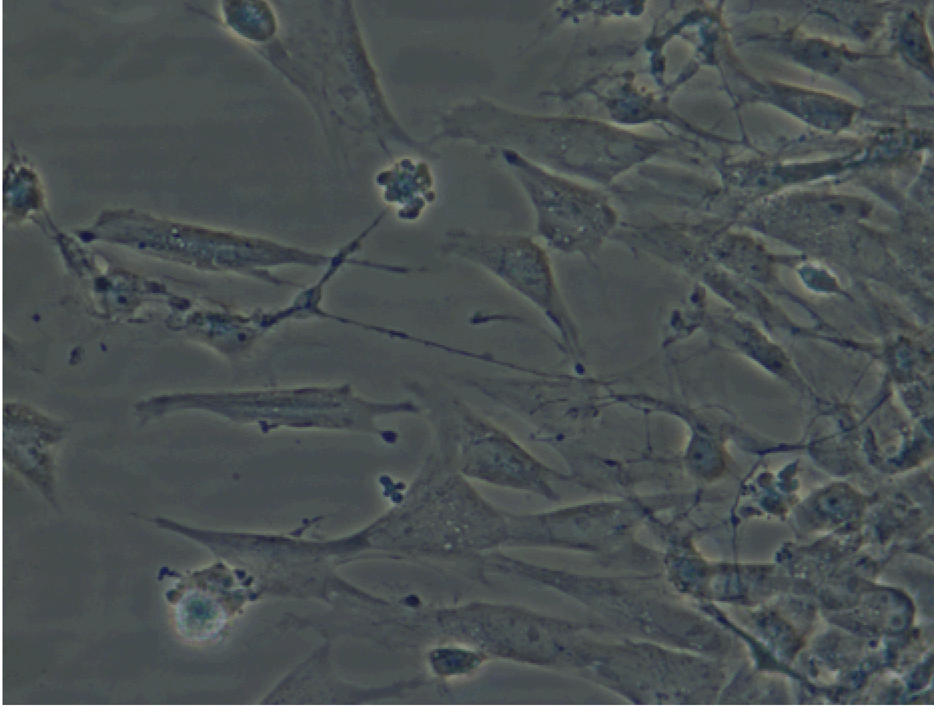
$$\frac{\text{Average brightness}_1}{\text{Average brightness}_2} = X \qquad \text{[Eqn 1]}$$

where brightness_1 denotes the lager value for the brighter image and brightness_2 denotes the smaller value for the dimmer image. Both images were set at 4.016 units of brightness, a ratio value calculated using Equation 1. Since color vision varies for each individual and disease may impair color vision, grayscale was chosen to highlight the contrast between light and dark areas reducing analysis errors that may arise due to variations in color vision. The range of grayscale is from 0-250, where 0 is absolute white and 250 is absolute black. The axon electrical charge was equated to the brightness. Axons in this study were defined as straight extensions off the cells. The measured extensions were chosen at random because the experimental and control treatment was consistent across the entire plate. The average gray value or brightness of the axons in each image (n=6) was calculated from histograms generated in ImageJ. The mean mitochondrial brightness and standard deviation is used to make comparisons between the experimental and control group.

Results

Mitochondrial cells were observed under transmitted light microscope and fluorescent microscope with Mitotracker. The intensity of light emitted by axonal mitochondria was captured. Axon length increased post-treatment with MeHg. The average length of axons in the control was 158.4 pixels. While, the average axon length for the experimental group was 219.25 pixels. Glia cells accounted for most of the cell growth as indicated by Figure 1 and Figure 2. The axons straight extensions amongst the cells were selected for and measures as axons. It was observed that there was an increase in apoptotic blebs in the group treated with +MeHg in DMEM compared to the control group.

A



B

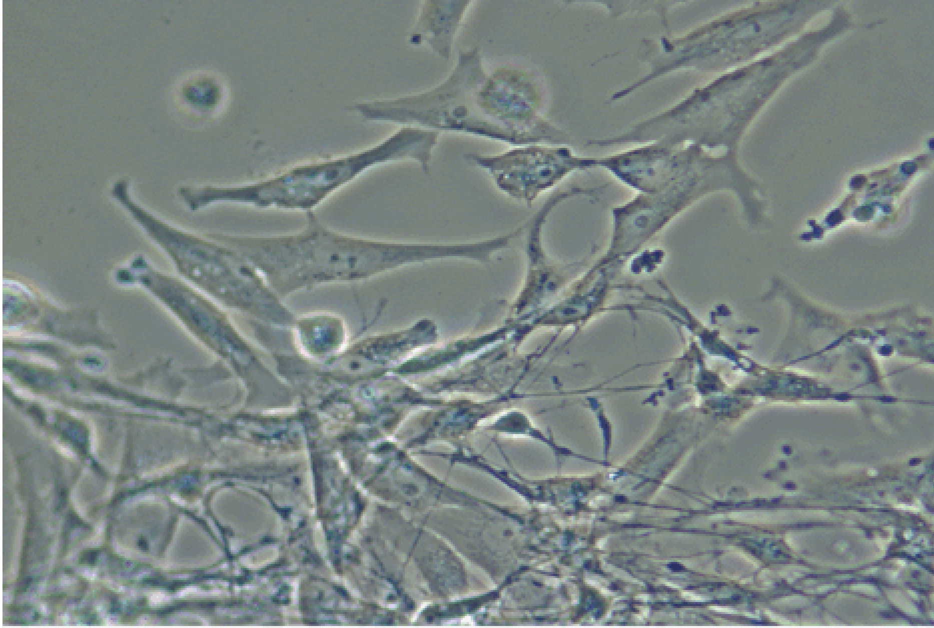
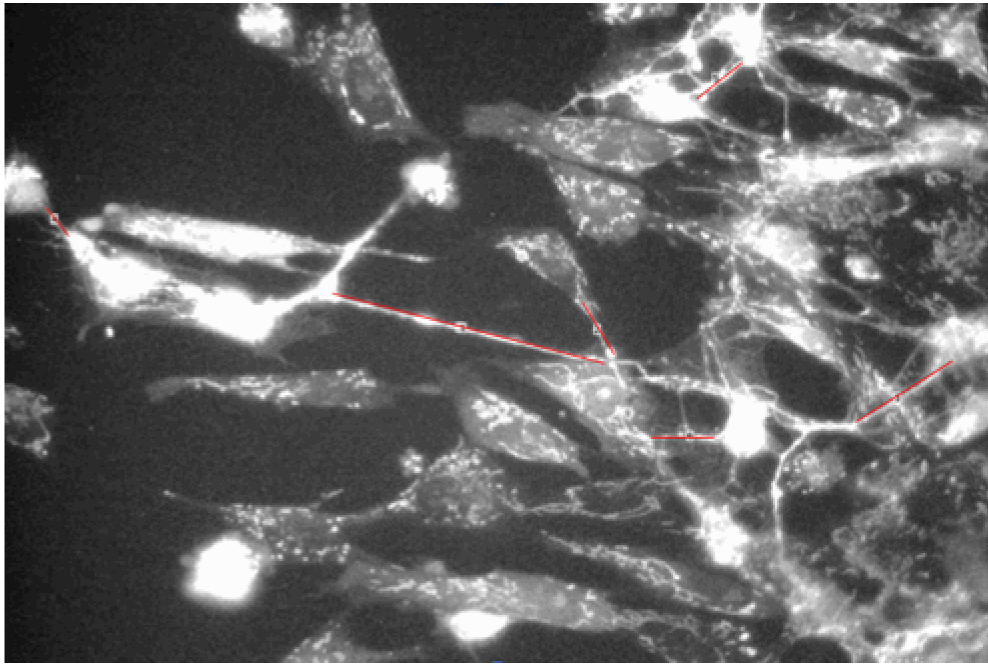


Figure 1: Image (A) shows the control axons exposed to -MeHg DMEM solution with transmitted light microscopy. Image (B) shows axons in experimental phase with treatment +MeHg DMEM solution using transmitted light microcopy. Note the width of the axons appear thinner after treatment in MeHg. Collaborated with Jenna Rocha.

A



B

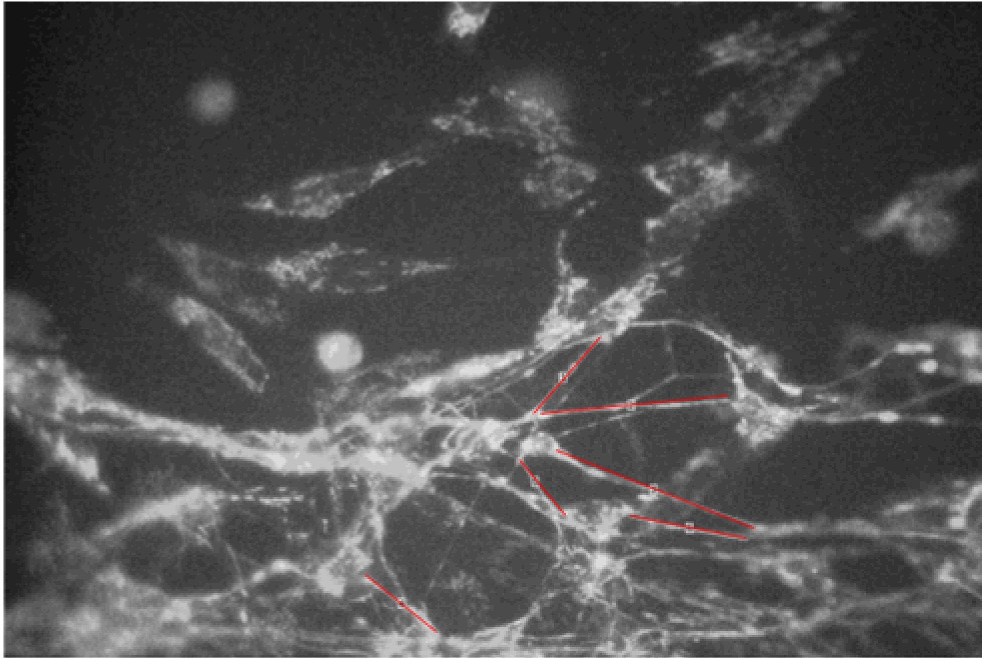


Figure 2: Image (A) captures control phase axons exposed to -MeHg DMEM solution with fluorescent Mitotracker (red); image (B) captures axons in experimental phase exposed to +MeHg DMEM solution with fluorescent Mitotracker (red) label. Note the axons in the experimental group were longer but the mitochondria emitted dimmer light than in control group. Collaborated with Jenna Rocha.

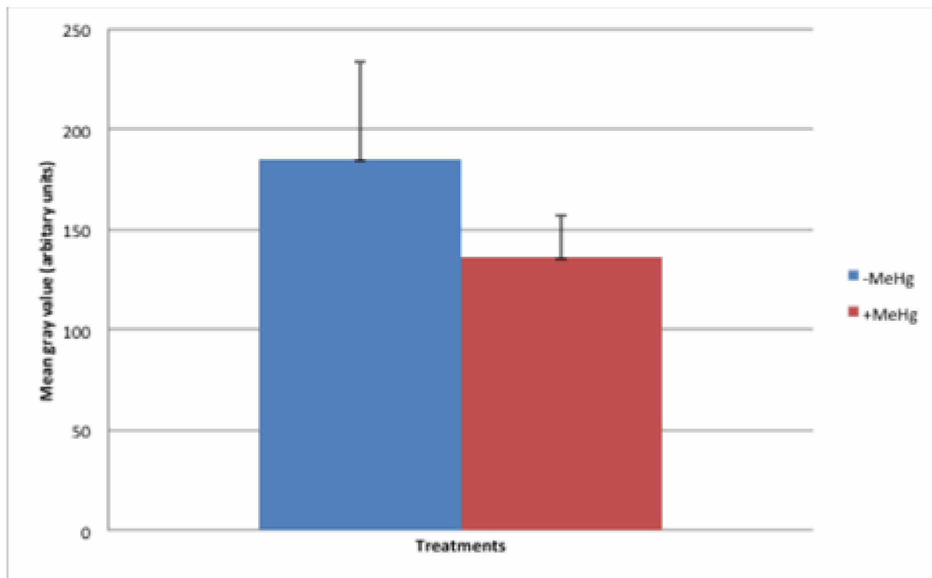


Figure 3: Mean Brightness of Axonal Mitochondria in methylmercury (CH_3Hg^+) shows average brightness of axons (n=6) in control and +MeHg treated solutions. Note the brightness value decreases with acute 40nM methylmercuy exposure.

The fluorescent images show that mitochondria concentration decreases in presence of methylmercury. The control group showed higher mean brightness of 185.5 ± 48 gray per pixels. The experimental group showed decreased mean brightness of 136.4 ± 21 gray per pixels. Figure 3 shows that there is significant different between mean gray value because the error bars do not overlap. This indicates p-value <0.05 and supports that there is different between control group and treatment with MeHg.

Discussion and Conclusion

The data gathered supports the hypothesis that axon mitochondrial brightness will decrease with exposure to methylmercury. The mitochondrial distribution is expected to be most concentrated in growing regions of the axon and dissipated when growth is inhibited (Morris and Hollenbeck, 1993). MeHg is shown to be an environmental factor that inhibits the trafficking of mitochondria to the axons. This decreases the density of mitochondria along the axon. The density of mitochondria in the axon is correlated to the brightness light emitted from Mitotracker with fluorescent

lighting. Lower concentration of mitochondria will emit dimmer light in summation. Reduced number of mitochondria, which are negatively charged, results in more positive overall voltage in the distal axon. Figure 1B supports that dimmer axon brightness is correlated to two factors, lower concentration of mitochondria in axons exposed to MeHg and a decrease in overall negativity in mitochondria that remain the axon. The mechanism that contributes to the results in this study can be inferred from other studies using methyl mercury. One study has indicated that methylmercury inhibits the electron transport system by binding to mitochondrial protein (Sone et. al., 1977). This resulted in a change in permeability in the mitochondria pore. The organelle becomes more permeable to K+ and the membrane potential is dissipated due to the imbalance.

Another consequence of MeHg exposure is that the movement of mitochondria is changed within the axon. The mitochondria contain bidirectional motor activity for anterograde and retrograde directions (Morris and Hollenbeck, 1993). The retrograde movement of mitochondria accommodates the stress induced by MeHg. Studies with neonatal rats have indicated that methyl mercury inhibits mitochondrial respiration by trapping electrons in the mitochondrial structure (O'Kusky, 1983). Trapped electrons cannot be used to convert ADP to ATP, which provides the energy needed for normal cellular functioning. Mitochondria have a higher concentration in regions of higher ATP. Therefore, MeHg decreases the production of ATP in growing regions and consequently may signal retrograde movement in mitochondria. As MeHg is introduced onto the axon, it triggers a signaling pathway that decreases ATP and promotes mitochondria movement away from axon, which further decreases ATP. With decrease mitochondria count and decrease energy production the axons eventually cease to function.

As shown by Figure 2, methylmercury has a significant impact at cellular level. It is less unclear the impact of the chemical on an organism. The same concentrations of MeHg may have different impact based on age. Future studies may look at the specific concentrations needed to impact change on the neurons and organism as a function on age. Also, other studies can look at the brightness of the soma and axons after treatment with methyl mercury. If this study is repeated, a larger sample size is suggested, as this is a possible limitation in this study.

References

Dreiem, A. (2005). The Effects of Methylmercury on Mitochondrial Function and Reactive Oxygen Species Formation in Rat Striatal Synaptosomes Are Age-Dependent. *Toxicological Sciences,* 87(1):156-62.

Dreim, A. & Seegal, R. (2007). Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent. *Neurotoxicology*: 28(4): 720-26.

Griffiths, E. (2000). Mitochondria — Potential Role in Cell Life and Death.*Cardiovascular Research,* 46(1) pp. 24-27.

Mannella, C.A. (2013). Mitochondrial Membranes, Structural Organization. *Encyclopedia of Biological Chemistry*, 145-48.

Morris, R. L., Hollenbeck, P. J. (1993). The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth. *Journal of Cell Science, 104,* 917-927.

Morris, R.L. (2014a). Primary Culture of Chick Embryonic Peripheral Neurons: Dissection. Retrieved from: http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_1_Dissection_2014.htm

Morris, R.L. (2014b). Primary Culture Of Chick Embryonic Peripheral Neurons 3: Observation and of Live Cells. Retrieved from: http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_2_ObserveLiveCells_2014.htm

Morris, R.L. (2014c). Primary Culture Of Chick Embryonic Peripheral Neurons 3: Staining and Observation of Live Cells. Retrieved from: http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_3_StainAndObserv_2014.htm

O'Kusky,J. (1983). Methylmercury poisoning of the developing nervous system: Morphological changes in neuronal mitochondria. *Acta Neuropathologica*, 61(2):116.

Sone, N., Larsstuvold, M.K., Kagawa, Y. (1997). Effect of methyl mercury on phosphorylation, transport, and oxidation in mammalian mitochondria.*Journal of Biochemistry*, 82(3): 859-68.

Verburg, J., and P. J. Hollenbeck. (2008).Mitochondrial Membrane Potential in Axons Increases with Local Nerve Growth Factor or Semaphorin Signaling. *Journal of Neuroscience,* 28(33): 8306-315.

Collaborated with Jenna Rocha.